Biochemical mechanism of flower color changes and their effects on pollinators in *Weigela* (Caprifoliaceae) and *Hibiscus* (Malvaceae)

タニウツギ属植物とフヨウ属植物における花色変化の生化学的機構と送粉昆虫に与える影響（英文）

Satoshi SHIMOKAWA

Department of Biological Sciences
Graduate School of Science and Engineering
Tokyo Metropolitan University
Minami-Osawa 1-1, Hachioji, Tokyo 192-0397, Japan

Tokyo Metropolitan University
2015

Contents

Summary

Flower color change is a character observed sporadically in several angiosperm taxa across 33 orders, 76 families, and 268 genera. These taxa change their flower color sequentially after flowering. However, the biochemical mechanisms involved in flower color change are poorly understood. In addition, the phylogenetic relationships among plant taxa with the flower color change character are not well documented. Therefore, one of the purposes of this study was to clarify the biochemical mechanisms of flower color change and evaluate the phylogenetic relationships among species that do and do not change their flower color.

Several studies have suggested that changing the flower color enhances the attraction of long-distance pollinators as these species retain their old flowers after the color change. Another effect is guiding short-distance pollinators to the flowers with a higher reproductive value by changing flower color. Another objective of this study was to examine the effects of changing the flower color on pollinators.

Plants in the genera *Weigela* and *Hibiscus* were used for the study. The genus *Weigela* (Caprifoliaceae) contains several species that change flower color. The colors of most *Weigela* species flowers remain red or pink, even after flowering. In contrast, four

Weigela species change the color of their flowers sequentially from white or yellow to red after flowering.

The genus *Hibiscus* (Malvaceae) also contains several species that change their flower color. The flowers of *Hibiscus hamabo*, *H. tiliaceus*, and *H. glaber*, which are distributed in Japan, change from yellow to orange or red after flowering. Most of the flavonoids contained in the petals of these species have not been identified, and a quantitative survey of flavonoids during changes in flower color has not been performed.

The flavonoids in the corollas of seven *Weigela* species and two hybrids, as well as their quantitative variations after flowering are characterized in Chapter I. The pigments from the corollas of fresh *W. hortensis* (ca. 124 g), *W. coraeensis* (ca. 107 g), *W. decora* (ca. 86 g), and *W. middendorffiana* (ca. 156 g) were extracted and identified. As a result, three anthocyanins and five flavonols were isolated from the corollas. The results suggest that the flower color changes in *Weigela* are caused by increased anthocyanin content. Moreover, the compositions of the anthocyanins and flavonols in the corolla were very similar among most *Weigela* species; however, the compositions of these pigments in *W. maximowiczii* and *W. middendorffiana* were very different from those in the other species.

In Chapter II, the phylogenetic relationships among 15 *Weigela* species that do

and do not change flower color are evaluated using molecular phylogenetic analyses based on the nucleotide sequences of the internal transcribed spacer region. The molecular phylogenetic tree analysis showed that the four *Weigela* species that change flower color after flowering were scattered among several clades. Accordingly, these data suggest that the flower color change character in *Weigela* was derived independently several times in each lineage. Additionally, *W. middendorffiana* and *W. maximowiczii*, which have peculiar yellow flowers, were revealed to be sister to the clade of the other *Weigela* species.

The effects of flower color change in *W. coraeensis* were examined in Chapter III based on their pollinators. The flowers of *W. coraeensis* change from white to red, whereas the flowers of *W. coraeensis* f. *alba* remain white, even after flowering. In other words, *W. coraeensis* has bicolor (white and red) flowers on each plant, whereas *W. coraeensis* f. *alba* has white monochrome flowers. Two hypotheses were tested in this study: 1) *W. coraeensis* with bicolor flowers will attract pollinators more effectively than that of *W. coraeensis* f. *alba* with monochrome flowers; and 2) pollinators will selectively visit flowers before the color change (white flowers) compared to those after the color change (red flowers). As results, the workers of *Bombus ardens*, which is the primary *W. coraeensis* pollinator, preferred to visit *W. coraeensis* plants containing both red and white

flowers rather than visiting *W. coraeensis* f. *alba* plants with only white flowers. Therefore, the pollinators preferred visiting plants with bicolor flowers than those with monochrome flowers. Such pollinator behavior is expected to affect *W. coraeensis* pollination efficiency.

Quantitative variations in the flavonoids, including anthocyanins, in the petals of *H. hamabo*, *H. tiliaceus*, and *H. glaber* were examined in Chapter IV. As a result, an anthocyanin and four flavonols were isolated from the petals of the three species. Flavonol and anthocyanin contents in *H. hamabo*, *H. tiliaceus*, and *H. glaber* increased after flowering, indicating that the color changes in the flowers of these three *Hibiscus* species are caused by increases in flavonol and anthocyanin contents.

The biochemical mechanisms of the *Weigela* and *Hibiscus* flower color changes were elucidated by identifying the flower pigments and their quantitative variations after flowering. Besides, my data suggested that the character of flower color change in *Weigela* was independently obtained several times in each lineage, probably in different geographical areas. This study clarified that pollinators preferred to visit *W. coraeensis* plants with both red and white flowers rather than *W. coraeensis* f. *alba* plants with only white flowers, and that the pollinators of *W. coraeensis* selectively visited young white flowers rather than older red flowers of the plant species. These facts support the

hypothesis that changing the flower color increases pollination efficiency, and suggests why many angiosperm species have evolved the flower color change character.

General Introduction

Flower is reproductive organ of spermatophyte. Many angiosperm flowers have colorful petals and sepals, and attract animal pollinators (e.g. insects and birds). Such zoidiophilous flowers have evolved wide variety of flower colors according to the preferences of the pollinators so that these flowers can attract more pollinators (Faegri & van der Pijl 1966, Fenster et al. 2004). Moreover, they also evolved flower color change possibly interating with pollinators so as to attract pollinators more efficiently to the flowers (Chittka 1996). Flower color change after flowering is one of the important characters in these contexts. Several types of flower color change exist, for example, some change color of whole petal, and others change color of only nectar guide in the petal. Flower color change is observed in various angiosperm taxa across 33 orders, 76 families, and 268 genera (Weiss 1995).

Flower color change has several effects on pollinators, such as enhancing attraction of long-distance pollinators by retaining old flowers after the color change (Gori 1983, Casper & La Pine 1984, Cruzan et al. 1988, Gori 1989, Delph & Lively 1989, Weiss 1991, Niesenbaum et al. 1999, Oberrath & Böhning-Gaese 1999). Moreover, the quantity of nectar produced differs before and after the flower color change in several

plants that change flower color. In fact, the flowers of *Cryptantha humilis* (Boraginaceae) produce large quantities of nectar before a color change; however, they produce little nectar after the color change (Casper & La Pine 1984). The visitation frequency of pollinators to flowers after a color change is lower than that before a color change (Casper & La Pine 1984). Thus, pollinators recognize flower color change, and they selectively visit flowers before a color change because there is a greater reward.

The flower colors of most plants develop because of the presence of flavonoids, carotenoids, chlorophylls, and betareins. Among these pigments, flavonoids are widely distributed over the plant kingdom and reveal a variety of colors including red, blue, and yellow; they also have an absorption maximum in the UV-A region. More than 8,150 types of flavonoids have been reported in plants (Anderson & Markham 2006). Flower color change is caused by qualitative and quantitative changes in these pigments (Ishikura 1982, Amrhein & Frank 1989, Weiss 1995, Farzad et al. 2002). However, a quantitative survey of flower pigments during flower color changes has been rarely performed. Therefore, the biochemical mechanisms of flower color change are poorly understood.

The genus *Weigela* (Caprifoliaceae) contains several species that change flower color. *Weigela* consists of approximately 12 species, distributed in northeast Asia, and the highest species diversity occurs in Japan and Korea (Hara 1983). Most *Weigela* spp.

flowers remain red or pink after flowering. In contrast, four *Weigela* spp. sequentially change flower color from white or yellow to red after flowering (Hara 1983, Chang 1997). Most of the flavonoids contained in the corollas of *Weigela* spp. have not yet been identified, and a quantitative survey of flavonoids in *Weigela* flowers during flower color changes has never been performed. In addition, the phylogenetic positions of the *Weigela* spp. that change flower color after flowering remain unclear.

W. coraeensis change the color of their flowers from white to red, whereas *W. coraeensis f. alba* flowers remain white after flowering. In other words, *W. coraeensis* has white and red flowers on each plant, whereas *W. coraeensis f. alba* has only white flowers. Therefore, we hypothesized that the contrast of bicolor flowers is more visually appealing than monochrome flowers, even for long-distance pollinators. In addition, we hypothesized that pollinators prefer to visit plants with bicolor flowers rather than those with monochrome flowers. Flowers of *W. coraeensis* produce larger quantities of nectar before than those after the color change (Suzuki et al. 2014). Previous studies reported that pollinators recognize the flower color change and selectively visit flowers before the color change to collect the additional nectar. Thus, it is expected that pollinators will visit white flowers more frequently on the first day of flowering than red flowers several days after flowering.

The genus *Hibiscus* (Malvaceae) also contains several species that change their flower color after flowering. *Hibiscus* consists of over 200 species, which are widely distributed in the tropics, subtropics, and partly in the temperate regions in the Northern Hemisphere. *H. hamabo*, *H. tiliaceus*, and *H. glaber* are distributed in Japan and change their flower color from yellow to orange or red after flowering. Most of the flavonoids contained in the petals of these species have not been identified, and a quantitative survey of flavonoids during flower color changes has not been performed.

The organization of this thesis is as follows. The flavonoids contained in the corollas of seven species and two hybrids of *Weigela* are characterized in Chapter I. The content of each flavonoid during flower color changes was quantified to clarify the biochemical mechanisms. Molecular phylogenetic analyses based on the nucleotide sequences of the internal transcribed spacer (ITS) region of nuclear ribosomal DNA are described in Chapter II. The phylogenetic relationships among *Weigela* spp. that do and do not change their flower color were evaluated. The effects of the change in the flower color of *W. coraeensis* on pollinators were examined in Chapter III. Two hypotheses were tested: 1) *W. coraeensis* with bicolor flowers will more effectively attract pollinators than *W. coraeensis f. alba* with monochrome flowers and 2) pollinators will more frequently visit flowers before the color change (white flowers) than those visiting

after the color change (red flowers). The flavonoids in the petals of *H. hamabo*, *H. tiliaceus*, and *H. glaber* are characterized and their quantitative variations after flowering are described in Chapter IV.

Chapter I.

Flower color changes in *Weigela* species: further characterization and quantitative variation of anthocyanins and flavonols

Introduction

Most flowering plants maintain their flower color after flowering, but some species change their colors sequentially after flowering (Weiss 1995). In these species, flower color change is caused by a quantitative change of the pigments in the petals in many cases. For example, *Viola cornuta* cv. Yesterday, Today, and Tomorrow changes its flower color from white to blue after flowering. In this plant, the anthocyanin, malvidin, increases, whereas the flavonol, myricetin, does not increase after flowering (Farzad et al. 2002). *Hibiscus mutabilis* var. *versicolor* changes its flower color from white to pink during a day. Two anthocyanins, cyanidin 3-*O*-sambubioside and cyanidin 3-*O*-glucoside, are synthesized only in the pink petals of this species, showing that they are produced during flower color change after flowering (Ishikura 1982; Amrhein & Frank 1989). Similarly, *Gossypium hirsutum* changes its flower color from yellow to pink (Muthusamy & Jayabalan 2011). Two anthocyanins, cyanidin 3-*O*-xylosylglucoside and cyanidin 3-*O*-glucoside, are synthesized in the petals of this species (Ismailov et al. 1994). Because

these anthocyanins are red pigments, it was proposed that they are produced after flowering.

Plant taxa that change their flower colors are widespread across 268 genera and 76 families (Weiss 1995). However, because quantitative surveys of the pigments of flower color change are hardly performed, the biochemical mechanisms of flower color change are not well elucidated.

Weigela Thunb. (Caprifoliaceae) consists of approximately 12 species distributed in Northeast Asia with the highest species diversity in Japan and Korea (Hara 1983). Plants of *Weigela* are deciduous shrubs and have long corollas (2–4 cm long; Hara 1983, Iwatsuki et al. 1993). Flower colors of the genus *Weigela* are mostly red or pink, but those of *W. middendorffiana* and *W. maximowiczii* are yellow. In four *Weigela* species, flower color changes sequentially after flowering. The colors of *W. decora*, *W. coraeensis*, and *W. japonica* change from white to red in the whole corollas, and similarly, those of *W. subsessilis* change from yellow to red in the whole corollas (Hara 1983, Chang 1997).

Among the pigments contained in *Weigela* corollas, flavonoids, such as kaempferol glycoside, quercetin 3-*O*-xyloside, quercetin 3-*O*-glucoside, quercetin 3-*O*-rutinoside, apigenin 7-*O*-glucoside, apigenin 7-*O*-rutinoside, and luteolin 7-*O*-glucoside

have been reported (Chang 1997). An anthocyanin, cyanidin 3-*O*-glucoside is synthesized in red corollas during the color change from yellow to red in *W. subsessilis* (Chang 1997), showing that the anthocyanin is produced during flower color change after flowering.

However, most of the flavonoids contained in the corollas of *Weigela* have not yet been identified, and flavonoid survey has been performed only in the following five species distributed or cultivated in Korea: *W. florida*, *W. praecox*, *W. hortensis*, *W. coraeensis*, and *W. subsessilis* (Chang 1997). No quantitative survey of the pigments including anthocyanin and other flavonoids in *Weigela* flowers during flower color change has been performed. In this chapter, I describe the further characterization and quantitative variation of flavonoids including anthocyanins in the corollas of seven species and two hybrids of *Weigela* after flowering.

Materials and Methods

Plant materials

Fresh corolla samples of seven species and two varieties of *Weigela* were used as materials (Table 1). Plant materials of these species were collected on May - June in 2009 and 2011.

General

Preparative paper chromatography (PPC) and analytical cellulose TLC were performed using the following three solvent systems: BAW (*n*-BuOH/HOAc/H_2O, 4:1:5, upper phase), 15% HOAc, and BEW (*n*-BuOH/EtOH/H_2O, 4:1:2.2). Preparative HPLC was performed on an *L*-column 2 ODS column (I.D. 10 × 250 mm, Chemicals Evaluation and Research Institute, Japan) at a flow rate of 1.5 ml min^{-1}, with detection at 350 nm (flavonols) and 530 nm (anthocyanin) and elution with MeCN/HOAc/H_2O/H_3PO_4 (6:8:83:3) for anthocyanins and MeCN/H_2O/H_3PO_4 (20:80:0.2) for flavonols. UV spectra were recorded on a Shimadzu MPS-2000 multipurpose recording spectrophotometer (220–500 nm) following Mabry et al. (1970). LC–MS was measured using an *L*-column 2 ODS column (I.D. 2.1 × 100 mm, Chemicals Evaluation and Research Institute) at a flow rate of 0.2 ml min^{-1}, eluting

with MeCN/H_2O/HCOOH (8:87:5) for anthocyanins and MeCN/H_2O/HCOOH (20:75:5) for flavonols, ESI^+ 4.5 kV, ESI^- 3.5 kV, 250°C. Isolated flavonoid glycosides were hydrolyzed with 12% HCl for 30 min in a boiling water bath. After cooling, the solution was shaken with diethyl ether, and the aglycones (diethyl ether phase) and sugars (mother liquor) were obtained.

Extraction and separation

Flower pigments were extracted with HCOOH/MeOH (8:92) from fresh corollas of *W. hortensis* (approximately 124 g), *W. coraeensis* (approximately 107 g), *W. decora* (approximately 86 g), and *W. middendorffiana* (ca. 156 g), respectively. After concentration, the extracts were fractionated by PPC using BAW and 15% HOAc and then BEW. The isolated flavonoids were purified on Sephadex LH-20 column chromatography using MeOH/HOAc/H_2O (70:5:25) for anthocyanins and 70% MeOH for other flavonoids. The isolated flavonoids were further purified by preparative HPLC.

Authentic standards

Authentic cyanidin 3-*O*-galactoside was obtained from the fruit skins of *Fatsia japonica* (Hayashi 1939), and cyanidin 3-*O*-glucoside was obtained from the leaves of *Acer palmatum* var. *matsumurae* (Hattori & Hayashi 1937). Authentic quercetin 3-*O*-glucoside-7-*O*-rhamnoside, quercetin 3-*O*-rutinoside, quercetin 3-*O*-galactoside, and quercetin 3-*O*-glucoside were obtained from the leaves of *Cornus multinervosa* (Iwashina & Hatta 1994), the leaves of *Osyris alba* (Iwashina et al. 2008), the flowers of *Notocactus ottonis* (Iwashina et al. 1982), and the leaves of *Phytolacca americana* (Iwashina & Kitajima 2009), respectively.

Identification

Flavonoids were identified by UV spectroscopy, LC–MS, acid hydrolysis, and HPLC comparison with authentic samples.

Quantitative HPLC analysis

For quantitative analysis, five sets of fresh corollas (each 0.2 g) were collected on the fifth day after flowering from five different individuals of each of *W. hortensis*, *W. floribunda*, *W. coraeensis*, and *W. decora*. Anthocyanins and flavonols were

extracted from the collected fresh corollas with 3 ml of HCOOH/MeOH (8:92) and MeOH, respectively. After filtration, the extracts were analyzed by HPLC. Relative anthocyanin and flavonol contents were determined by the peak area of each compound on HPLC chromatograms.

Result

Identification of flavonoids

Three anthocyanins and five flavonols were isolated from the corollas of the genus *Weigela*. Anthocyanins such as cyanidin 3-*O*-galactoside (**A1**), cyanidin 3-*O*-glucoside (**A2**), and cyanidin 3-*O*-rutinoside (**A3**) and flavonols such as quercetin 3-*O*-glucoside-7-*O*-rhamnoside (**F1**), quercetin 3-*O*-rutinoside (**F3**), quercetin 3-*O*-galactoside (**F4**), and quercetin 3-*O*-glucoside (**F5**) were identified based on their UV spectrl properties, LC–MS, and HPLC comparison with authentic standards. Quercetin 3-*O*- xylosylgalactoside (**F2**) was characterized by UV spectroscopy, LC–MS, and acid hydrolysis. Each flavonoid was identified by the following data and Tables 4–13.

Cyanidin 3-*O*-galactoside (**A1**)

TLC (R_f): 0.25 (BAW), 0.28 (15% HOAc), 0.18 (BEW)

Color: Visible–red purple

HPLC (R_t): 5.3 min

UV λ_{max} (nm): 0.01% HCl-MeOH| 281, 332sh, 530; +$AlCl_3$| 312, 387sh, 566

E_{acid}/E_{max} (%): 66, $E440/E_{max}$ (%): 26

LC–MS: *m*/*z* 449 $[M+H]^+$ (molecular ion peak, cyanidin + 1 mol galactose), *m*/*z* 287 [M–

162+H]$^+$ (fragment ion peak, cyanidin).

Cyanidin 3-*O*-glucoside (**A2**)

TLC (R_f): 0.23 (BAW), 0.17 (15% HOAc), 0.16 (BEW)

Color: Visible—red purple

HPLC (R_t): 6.1 min

UV λ_{max} (nm): 0.01% HCl-MeOH| 281, 531; +$AlCl_3$| 313, 369sh, 569

E_{acid}/E_{max} (%): 59, $E440/E_{max}$ (%): 25

LC–MS: *m*/*z* 449 [M+H]$^+$ (molecular ion peak, cyanidin + 1 mol glucose), *m*/*z* 287 [M–162+H]$^+$ (fragment ion peak, cyanidin).

Cyanidin 3-*O*-rutinoside (**A3**)

TLC (R_f): 0.28 (BAW), 0.35 (15% HOAc), 0.24 (BEW)

Color: Visible—red purple

HPLC (R_t): 8.5 min

UV λ_{max} (nm): 0.01% HCl-MeOH| 280, 367sh, 531; +$AlCl_3$| 313, 361sh, 568

E_{acid}/E_{max} (%): 63, $E440/E_{max}$ (%): 22

LC–MS: *m*/*z* 595 [M+H]$^+$ (molecular ion peak, cyanidin + 1 mol glucose and rhamnose),

m/*z* 449 $[M–146+H]^+$ (fragment ion peak, cyanidin + 1 mol glucose), *m*/*z* 287 $[M–308+H]^+$ (fragment ion peak, cyanidin).

Quercetin 3-*O*-glucoside-7-*O*-rhamnoside (**F1**)

TLC (R_f): 0.35 (BAW), 0.45 (15% HOAc), 0.20 (BEW)

Color: UV (365 nm)—dark purple, UV/NH_3—yellow

HPLC (R_t): 4.1 min

UV λ_{max} (nm): MeOH| 256, 358; +NaOMe| 270, 396 (inc.); +$AlCl_3$| 275, 440; +$AlCl_3$/HCl| 270, 365sh, 403; +NaOAc| 262, 410; +NaOAc/H_3BO_3| 261, 382.

LC–MS: *m*/*z* 611 $[M+H]^+$ (molecular ion peak, quercetin + each 1 mol glucose and rhamnose), *m*/*z* 449 $[M–162+H]^+$ (fragment ion peak, quercetin + 1 mol rhamnose), *m*/*z* 303 $[M–308+H]^+$ (fragment ion peak, quercetin).

Quercetin 3-*O*-xylosylgalactoside (**F2**)

TLC (R_f): 0.40 (BAW), 0.64 (15% HOAc), 0.48 (BEW)

Color: UV (365 nm)—dark purple, UV/NH_3—yellow

HPLC (R_t): 5.1 min

UV λ_{max} (nm): MeOH| 256, 302sh, 354; +NaOMe| 271, 402 (inc.); +$AlCl_3$| 274, 433;

+$AlCl_3$/HCl| 270, 299sh, 360, 402; +NaOAc| 274, 326sh, 388; +NaOAc/H_3BO_3| 261, 295sh, 374.

LC–MS: *m*/*z* 597 $[M+H]^+$ (molecular ion peak, quercetin + 1 mol galactose and xylose), *m*/*z* 465 $[M–132+H]^+$ (fragment ion peak, quercetin + 1 mol galactose), *m*/*z* 303 $[M–294+H]^+$ (fragment ion peak, quercetin).

Quercetin 3-*O*-rutinoside (**F3**)

TLC (R_f): 0.37 (BAW), 0.60 (15% HOAc), 0.38 (BEW)

Color: UV (365 nm)—dark purple, UV/NH_3—yellow

HPLC (R_t): 6.5 min

UV λ_{max} (nm): MeOH| 257, 358; +NaOMe| 272, 328sh, 410 (inc.); +$AlCl_3$| 275, 434; +$AlCl_3$/HCl| 270, 300sh, 402; +NaOAc| 273, 325sh, 399; +NaOAc/H_3BO_3| 262, 295sh, 379.

LC–MS: *m*/*z* 611 $[M+H]^+$ (molecular ion peak, quercetin + each 1 mol glucose and rhamnose), *m*/*z* 465 $[M–146+H]^+$ (fragment ion peak, quercetin + 1 mol glucose), *m*/*z* 303 $[M–308+H]^+$ (fragment ion peak, quercetin).

Quercetin 3-*O*-galactoside (**F4**)

TLC (R_f): 0.44 (BAW), 0.27 (15% HOAc), 0.55 (BEW)

Color: UV (365 nm)—dark purple, UV/NH_3—yellow

HPLC (R_t): 7.1 min

UV λ_{max} (nm): MeOH| 257, 360; +NaOMe| 272, 328sh, 411 (inc.); +$AlCl_3$| 275, 338sh, 436; +$AlCl_3$/HCl| 269, 300sh, 403; +NaOAc| 273, 323sh, 392; +NaOAc/H_3BO_3| 261, 297sh, 378.

LC–MS: *m*/*z* 465 $[M+H]^+$ (molecular ion peak, quercetin + 1 mol galactose), *m*/*z* 303 $[M–162+H]^+$ (fragment ion peak, quercetin).

Quercetin 3-*O*-glucoside (**F5**)

TLC (R_f): 0.54 (BAW), 0.30 (15% HOAc), 0.66 (BEW)

Color: UV (365 nm)—dark purple, UV/NH_3—yellow

HPLC (R_t): 7.4 min

UV λ_{max} (nm): MeOH| 257, 360; +NaOMe| 272, 328sh, 411 (inc.); +$AlCl_3$| 275, 338sh, 434; +$AlCl_3$/HCl| 269, 299sh, 366, 401; +NaOAc| 264, 382; +NaOAc/H_3BO_3| 262, 380.

LC–MS: *m*/*z* 465 $[M+H]^+$ (molecular ion peak, quercetin + 1 mol glucose), *m*/*z* 303 $[M–162+H]^+$ (fragment ion peak, quercetin).

Distribution of anthocyanins

Flower anthocyanins of the genus *Weigela* were surveyed by HPLC. Qualitative HPLC analysis showed that the anthocyanin compositions of all the examined species of the genus *Weigela* except *W. middendorffiana* and *W. maximowiczii* were very similar (Table 2). The corollas of *W. middendorffiana* contained cyanidin 3-*O*-glucoside (**A2**) and cyanidin 3-*O*-rutinoside (**A3**). In particular, cyanidin 3-*O*-rutinoside (**A3**) was found only in this species (Table 2). No anthocyanins were detected in the corollas of *W. maximowiczii* (Table 2). Among the anthocyanins detected in the corollas, cyanidin 3-*O*-galactoside (**A1**) was the major compound (over 60%) in all surveyed species of the genus *Weigela* but *W. middendorffiana* and *W. maximowiczii* (Table 2). Cyanidin 3-*O*-glucoside (**A2**) was the major compound (over 60%) in *W. middendorffiana* (Table 2).

Distribution of flavonols

With respect to flavonols which found in this survey, quercetin 3-*O*-galactoside (**F4**) and quercetin 3-*O*-glucoside (**F5**) were found in almost species of *Weigela* (Table 3). Quercetin 3-*O*-xylosylgalactoside (**F2**) was also found in the three species and one hybrid (Table 3). Quercetin 3-*O*-glucoside-7-*O*-rhamnoside (**F1**) was detected only in *W. middendorffiana* as major compound (over 50%) (Table 3), and quercetin 3-*O*-rutinoside

(**F3**) was detected in *W. middendorffiana* and *W. maximowiczii* (Table 3). Quercetin 3-*O*-galactoside (**F4**) was the major compound (over 50%) in most species of the genus *Weigela* (Table 3).

Quantitative variation in total amounts of anthocyanins and flavonols

Contents of anthocyanins increased dramatically after flowering in *W. coraeensis* and *W. decora* but hardly changed in *W. hortensis* and *W. floribunda* (Figure 1). Contents of the flavonols hardly changed after flowering in *W. coraeensis*, *W. decora*, *W. hortensis*, and *W. floribunda* (Figure 2).

Discussion

Identification of flavonoids

Cyanidin 3-*O*-glucoside (**A2**), quercetin 3-*O*-rutinoside (**F3**), and quercetin 3-*O*-glucoside (**F5**) have already been reported from *W. florida*, *W. praecox*, and *W. subsessilis* (Chang 1997). Among the flavonoids of the *Weigela* species surveyed in this study, kaempferol glycoside, quercetin 3-*O*-xyloside, apigenin 7-*O*-glucoside, apigenin 7-*O*-rutinoside, and luteolin 7-*O*-glucoside have been reported from flowers of *Weigela* species (Chang 1997). However, these flavonoids were not isolated from these species in this survey. Quercetin 3-*O*-rutinoside (**F3**) has been reported from *W. hortensis* and *W. coraeensis* (Chang 1997), but this flavonol was not isolated from these species in this survey. In contrast, cyanidin 3-*O*-galactoside (**A1**), quercetin 3-*O*-glucoside-7-*O*-rhamnoside (**F1**), quercetin 3-*O*-xylosylgalactoside (**F2**), and quercetin 3-*O*-galactoside (**F4**) were found in *Weigela* for the first time in this study.

Distribution of anthocyanins and flavonols

In this study, no anthocyanins were detected in corollas of *W. maximowiczii* (Table 2). Anthocyanins act as red pigments in corollas. *W. maximowiczii* has greenish yellow or yellowish white flowers, perhaps because the flowers of the species lack

anthocyanins (Hara 1983). That author speculated that these flower colors are due to quercetin glycoside. In contrast, although the flower color in the corollas of *W. middendorffiana* is pale yellow (Hara 1983), the corollas of this species contained red anthocyanins, cyanidin 3-*O*-glucoside (**A2**) and cyanidin 3-*O*-rutinoside (Table 2). The nectar guide, which is a part of the corolla of *W. middendorffiana*, changes color from pale yellow to red after flowering. We thus expect that anthocyanins or red pigments are contained in the corollas of this species.

Anthocyanin and flavonol composition contained in the corolla was almost same with *W. hortensis*, *W. floribunda*, *W. coraeensis*, *W. decora*, *W. japonica*, *Weigela* × *fujisanensis* f. *versicolor*, and *W. amabilis* (Tables 2 and 3). Of these species, *W. coraeensis*, *W. decora*, and *W. japonica* change their flower color after flowering, whereas *W. hortensis*, *W. floribunda*, *Weigela* × *fujisanensis* f. *versicolor*, and *W. amabilis* maintain their flower color after flowering. The qualitative difference in flavonoids may thus not be associated with the flower color change in *Weigela*.

Quantitative variation in total amounts of anthocyanins and flavonols

In the corollas of *W. coraeensis* and *W. decora*, contents of anthocyanins increased dramatically after flowering, but those of flavonols hardly changed (Figures 1

and 2). In contrast, in the corollas of *W. hortensis* and *W. floribunda*, contents of both anthocyanins and flavonols hardly changed after flowering (Figures 1 and 2). The flower colors of *W. coraeensis* and *W. decora* change sensationally from white to red after flowering, whereas flowers of *W. hortensis* and *W. floribunda* remain pale pink or red even after flowering (Hara 1983). We infer that flower color changes in the genus *Weigela* are caused by increases in anthocyanin.

The total amount of anthocyanins found in the corollas of *W. coraeensis* was almost the same as those of *W. hortensis* (Figure 1). Thus, the composition of anthocyanins was already fixed even just after flowering in the corollas of *W. hortensis*, but began to change after flowering in the corollas of *W. coraeensis*. In other words, species which were changed flower color in the genus *Weigela* are started anthocyanin synthesize in later stages than species that do not change flower color.

Table 1. *Weigela* taxa used in this study and their collection sites and dates.

Taxa	Collection sites and dates
Weigela middendorffiana	Cultivated in Botanic Garden, Hokkaido University, Sapporo, Hokkaido, Japan, June 9, 2010
W. maximowiczii	Cultivated in Nikko Botanical Gardens, Graduate School of Science, The University of Tokyo, Tochigi Pref. , Japan, June 4, 2010
W. hortensis	Cultivated in Tsukuba Botanical Garden, National Museum of Nature and Sciense, Tsukuba, Ibaraki Pref., Japan, May, 2009
W. coraeensis	Ainohara, Atami, Shizuoka Pref., Japan, May, 2009
W. decora	Cultivated in Tsukuba Botanical Garden, National Museum of Nature and Sciense, Tsukuba, Ibaraki Pref., Japan, May, 2009
W. floribunda	Cultivated in Tsukuba Botanical Garden, National Museum of Nature and Sciense, Tsukuba, Ibaraki Pref., Japan, May 11, 2009
Weigela×*fujisanensis* f. *versicolor*	Cultivated in Nikko Botanical Gardens, Graduate School of Science, The University of Tokyo, Nikko, Tochigi Pref., Japan, June 5, 2010
W. amabilis	Cultivated in Tokyo Metropolitan Medicinal Plant Garden, Tokyo, Japan, Jun 29, 2010
W. japonica	Cultivated in The Kochi Prefectural Makino Botanical Garden, Kochi Pref., Japan, June 4, 2010

All the plant samples used in this study was collected by S. Shimokawa.

Table 2. Distribution of anthocyanins in the corollas of seven species and two hybrids of *Weigela.*

Taxa	A1	A2	A3
W. middendorffiana		+++	++
W. maximowiczii			
W. hortensis	+++	++	
W. floribunda	+++	++	
W. coraeensis	+++	++	
W. decora	+++	++	
W. japonica	+++	++	
Weigela×fujisanensis f. versicolor	+++	++	
W. amabilis	+++	++	

A1 = cyanidin 3-*O*-galactoside, A2 = cyanidin 3-*O*-glucoside, cyanidin 3-*O*-rutinoside. +++ = > 60% of the total anthocyanins, ++ = from 25 to 10%.

Table 3. Distribution of flavonols in the corollas of seven species and two hybrids of *Weigela*.

Taxa	F1	F2	F3	F4	F5
W. middendorffiana	+++		++		+
W. maximowiczii			+	+	++
W. hortensis		+		++	++
W. floribunda				+++	++
W. coraeensis				+++	++
W. decora		+		+++	+
W. japonica		++		++	+
Weigela×fujisanensis f. versicolor				+++	+
W. amabilis		++		+++	++

F1 = Quercetin 3-*O*-glucoside-7-*O*-rhamnoside, F2 = quercetion 3-*O*-xylosylgalactoside, F3 = Quercetin 3-*O*-rutinoside, F4 = Quercetin 3-*O*-galactoside, F5 = Quercetin 3-*O*- glucoside. +++ = > 50% of the total anthocyanins, ++ = from 50 to 30%, + = 30 from to 10%.

Table 4. TLC and HPLC data of anthocyanidins, which were obtained by acid hydrolysis anthocyanins from the corollas of *Weigela hortensis*.

Anthocyanidins	Rf values			Colors	HPLC
	BAW	Forestal	BEW	(visible)	(Rt, min)
A1	0.72	0.40	0.45	light red purple	5.8
A2	0.72	0.40	0.45	light red purple	5.8
Authenic specimens:					
cyanidin	0.70	0.42	0.45	light red purple	5.8

BAW = *n*-BuOH/HOAc/H_2O (4:1:5, upper phase), 15%HOAc = HOAc/H_2O (15:85), BEW = *n*-BuOH/HOAc/H_2O (4:1:2.2), Rt = retention time.

Table 5. TLC and HPLC data of anthocyanidins, which were obtained by acid hydrolysis anthocyanins from the corollas of *W. middendorffiana*.

anthocyanidins	Rf values			Colors	HPLC
	BAW	Forestal	BEW	(visible)	(Rt, min)
A2	0.68	0.42	0.47	light red purple	5.8
A3	0.68	0.42	0.47	light red purple	5.8
Authenic specimens:					
cyanidin	0.68	0.43	0.47	light red purple	5.8

BAW = *n*-BuOH/HOAc/H_2O (4:1:5, upper phase), 15%HOAc = HOAc/H_2O (15:85), BEW = *n*-BuOH/HOAc/H_2O (4:1:2.2), Rt = retention time.

Table 6. TLC and HPLC data of the aglycones, which were obtained by acid hydrolysis of flavonols from the corollas of *W. coraeensis*.

Aglycones	Rf values			Colors		HPLC
	BAW	Forestal	BEW	UV	UV/NH3	(Rt, min)
F4	0.76	0.56	0.73	reddish brown	yellow	6.3
F5	0.77	0.54	0.72	reddish brown	yellow	6.3
Authenic specimens:						
quercetin	0.76	0.55	0.75	reddish brown	yellow	6.3

BAW = *n*-BuOH/HOAc/H_2O (4:1:5, upper phase), 15%HOAc = HOAc/H_2O (15:85),
BEW = *n*-BuOH/HOAc/H_2O (4:1:2.2), Rt = retention time.

Table 7. TLC and HPLC data of the aglycones, which were obtained by acid hydrolysis of flavonols from the corollas of *W. middendorffiana.*

Aglycones	Rf values			Colors		HPLC
	BAW	Forestal	BEW	UV	UV/NH3	(Rt, min)
F1	0.85	0.49	0.74	reddish brown	yellow	6.3
F3	0.85	0.50	0.73	reddish brown	yellow	6.3
Authenic specimens:						
quercetin	0.83	0.50	0.75	reddish brown	yellow	6.3

BAW = *n*-BuOH/HOAc/H_2O (4:1:5, upper phase), 15%HOAc = HOAc/H_2O (15:85),
BEW = *n*-BuOH/HOAc/H_2O (4:1:2.2), Rt = retention time.

Table 8. TLC and HPLC data of the aglycones, which were obtained by acid hydrolysis of flavonols from the corollas of *W. decora.*

Aglycones	Rf values			Colors		HPLC
	BAW	Forestal	BEW	UV	UV/NH3	(Rt, min)
F2	0.79	0.51	0.72	reddish brown	yellow	6.3
Authenic specimens:						
quercetin	0.8	0.52	0.74	reddish brown	yellow	6.3

BAW = *n*-BuOH/HOAc/H_2O (4:1:5, upper phase), 15%HOAc = HOAc/H_2O (15:85),

BEW = *n*-BuOH/HOAc/H_2O (4:1:2.2), Rt = retention time.

Table 9. PC data of glycosidic sugars, which were obtained by acid hydrolysis of anthocyanins from the corollas of *Weigela hortensis*.

Sugar	Rf values		Colors	Identity
	BBPW	BTPW		
A1	0.25	0.24	brownish yellow	galactose
A2	0.29	0.27	brownish yellow	glucose
Authentic specimens:				
glucose	0.30	0.31	brownish yellow	
galactose	0.29	0.26	brownish yellow	
allose	0.33	0.27	brownish yellow	
glucuronic acid	0.06	0.05	red	
	0.60	0.58	red	
arabinose	0.32	0.35	reddish brown	
xylose	0.43	0.38	reddish brown	
rhamnose	0.55	0.55	brownish yellow	

BBTW = *n*-BuOH/Benzene/Pyridine/H_2O (5:1:3:3),

BTPW = *n*-BuOH/Toluene/Pyridine/H_2O (5:1:3:3).

Table 10. PC data of glycosidic sugars, which were obtained by acid hydrolysis of anthocyanins from the corollas of *W. middendorffiana.*

Sugar	Rf values		Colors	Identity
	BBPW	BTPW		
A3	0.33	0.24	brownish yellow	glucose
	0.51	0.40	brownish yellow	rhamnose
Authentic specimens:				
glucose	0.32	0.24	brownish yellow	
galactose	0.31	0.22	brownish yellow	
allose	0.37	0.29	brownish yellow	
glucuronic acid	0.16	0.16	red	
	0.58	0.54	red	
arabinose			reddish brown	
xylose	0.36	0.28	reddish brown	
rhamnose	0.50	0.41	brownish yellow	

BBTW = *n*-BuOH/Benzene/Pyridine/H_2O (5:1:3:3),

BTPW = *n*-BuOH/Toluene/Pyridine/H_2O (5:1:3:3).

Table 11. PC data of glycosidic sugars, which were obtained by acid hydrolysis of flavonols from the corollas of *W. coraeensis*.

Sugar	Rf values		Colors	Identity
	BBPW	BTPW		
F4	0.22	0.23	brownish yellow	galactose
F5	0.28	0.26	brownish yellow	glucose
Authentic specimens:				
glucose	0.31	0.24	brownish yellow	
galactose	0.26	0.22	brownish yellow	
allose	0.28	0.28	brownish yellow	
glucuronic acid	0.10	0.09	red	
	0.58	0.56	red	
arabinose	0.33	0.31	reddish brown	
xylose	0.38	0.40	reddish brown	
rhamnose	0.51	0.49	brownish yellow	

BBTW = *n*-BuOH/Benzene/Pyridine/H2O (5:1:3:3),

BTPW = *n*-BuOH/Toluene/Pyridine/H2O (5:1:3:3).

Table 12. PC data of glycosidic sugars, which were obtained by acid hydrolysis of flavonols from the corollas of *W. middendorffiana.*

Sugar	Rf values		Colors	Identity
	BBPW	BTPW		
F1	0.25	0.19	brownish yellow	glucose
	0.47	0.50	brownish yellow	rhamnose
F3	0.25	0.20	brownish yellow	glucose
	0.47	0.50	brownish yellow	rhamnose
Authentic specimens:				
glucose	0.26	0.26	brownish yellow	
galactose	0.23	0.21	brownish yellow	
allose	0.26	0.26	brownish yellow	
glucuronic acid	0.08	0.06	red	
	-	-	red	
arabinose	0.36	-	reddish brown	
xylose	0.36	0.33	reddish brown	
rhamnose	0.47	0.49	brownish yellow	

BBTW = *n*-BuOH/Benzene/Pyridine/H2O (5:1:3:3),

BTPW = *n*-BuOH/Toluene/Pyridine/H2O (5:1:3:3).

Table 13. PC data of glycosidic sugars, which were obtained by acid hydrolysis of flavonols from the corollas of *W. decora.*

Sugar	Rf values		Colors	Identity
	BBPW	BTPW		
F2	0.24	0.21	brownish yellow	galactose
	0.41	0.39	reddish brown	xylose
Authentic specimens:				
glucose	0.28	0.26	brownish yellow	
galactose	0.23	0.21	brownish yellow	
allose	0.31	0.30	brownish yellow	
glucuronic acid	0.08	0.07	red	
	0.53	0.52	red	
arabinose	0.36	0.33	reddish brown	
xylose	0.42	0.38	reddish brown	
rhamnose	0.53	0.49	brownish yellow	

BBTW = *n*-BuOH/Benzene/Pyridine/H2O (5:1:3:3),

BTPW = *n*-BuOH/Toluene/Pyridine/H2O (5:1:3:3).

(a) **(b)**

(c-1) **(c-2)** **(c-3)**

(c-4) **(c-5)**

Figure 1. Photograph of *Weigela hortensis* showing (a) whole plant, (b) inflorescence, and flowers on the (c-1) first, (c-2) second, (c-3) third, (c-4) fourth, and (c-5) fifth day of flowering.

(a)

(b)

(c-1)

(c-2)

(c-3)

(c-4)

(c-5)

Figure 2. Photograph of *W. floribanda* showing (a) whole plant, (b) inflorescence, and flowers on the (c-1) first, (c-2) second, (c-3) third, (c-4) fourth, and (c-5) fifth day of flowering.

(a)

(b)

(c-1)

(c-2)

(c-3)

(c-4)

(c-5)

Figure 3. Photograph of *W. coraeensis* showing (a) whole plant, (b) inflorescence, and flowers on the (c-1) first, (c-2) second, (c-3) third, (c-4) fourth, and (c-5) fifth day of flowering.

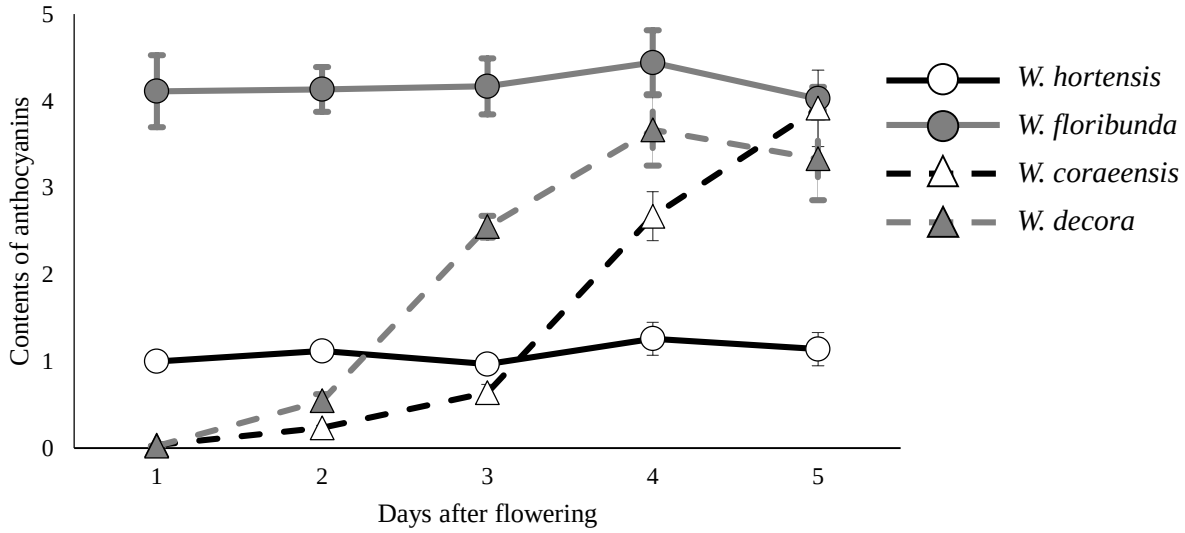


Figure 4. Quantitative variation of total amounts of anthocyanins in the corollas of *Weigela hortensis*, *W. floribunda*, *W. coraeensis*, and *W. decora* (mean ± 1 SE). The vertical axis shows the relative amounts of anthocyanins. The amounts are relative to the peak area of the first-day corolla after flowering of *W. hortensis*.

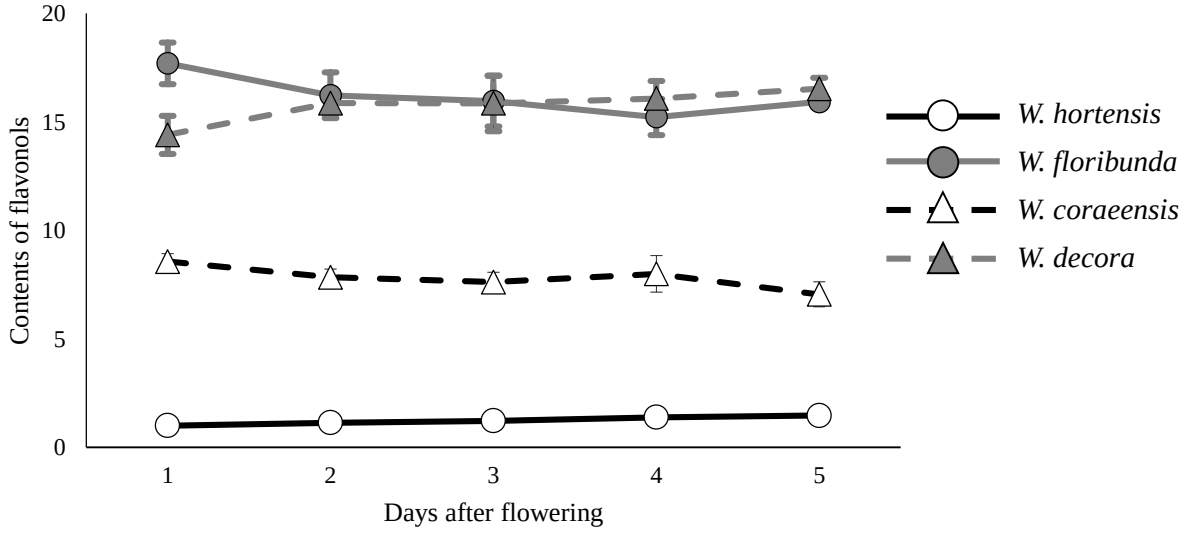


Figure 5. Quantitative variation of total amount of flavonols in the corollas of *Weigela hortensis*, *W. floribunda*, *W. coraeensis*, and *W. decora* (mean ± 1 SE). The vertical axis shows the relative amounts of flavonols. The amounts are relative to the peak area of the first-day corolla after flowering of *W. hortensis*.

(a)

(b)

(c-1)

(c-2)

Figure 6. Photographs of *Weigela middendorffiana* showing (a) whole plant, (b) inflorescence, (c-1) a flower before color change, and (c-2) a flower after color change.

Chapter II.

Phylogeny of *Weigela* (Caprifoliaceae) based on nuclear rDNA internal transcribed spacer sequences

Introduction

Weigela Thunb. (Caprifoliaceae) consists of approximately 12 woody species distributed in East Asia with the highest species diversity in Japan and Korea (Hara 1983). The most widely recognized taxonomical treatment of *Weigela* was published by Hara (1983), who divided the genus into four sections: *Weigela*, *Calysphyrum*, *Weigelastrum*, and *Calyptrostigma.* Among the four sections, *Weigelastrum* and *Calyptrostigma* were separated from *Weigela* and *Calysphyrum* in having hairy and connivent anthers and yellow corollas. Sections *Weigelastrum* and *Calyptrostigma* are monotypic, consisting only of *W. maximowiczii* and *W. middendorffiana*, respectively. Section *Weigela* differs from *Calysphyrum* in having a calyx deeply five lobed from the base and absence of seed wings (Hara 1983). Section *Calysphyrum* consists of three species: *W. florida*, *W. praecox*, and *W. toensis*. Section *Weigela* is much larger than the other three sections and contains most species of the genus.

Interspecific hybridizations have occurred relatively frequently in the genus

Weigela. Natural hybrids between various combinations of *W. decora*, *W. hortensis*, *W. coraeensis*, and *W. floribunda* are common by found in central Honshu, Japan (Hara 1983). These hybrids may complicate classification within the genus.

Flowers of the genus *Weigela* are mostly red or pink, but those of *W. middendorffiana* and *W. maximowiczii* are yellow. These species maintain their flower colors until the end of flowering (Hara 1983). In contrast, flower colors of four species of the genus change sequentially after flowering. The flower colors of *W. decora*, *W. coraeensis*, and *W. japonica* change from white to red in the whole corollas. Similarly, that of *W. subsessilis* changes from yellow to red again in the whole corolla (Hara 1983, Chang 1997).

Based on a molecular phylogenetic study using nucleotide sequences of the internal transcribed spacer (ITS) region of nuclear ribosomal DNA, sections *Weigelastrum* and *Calysphyrum* formed a monophyletic group (Kim & Kim, 1999), although the monophyly of each section was obscure and the relationship between the two sections was unclear. The phylogenetic positions of the species that change flower color after flowering are also unclear (Kim & Kim, 1999). In this chapter, I examined phylogenetic relationships among the species of the genus *Weigela* with flower color-changing characters using molecular phylogenetic analyses based on

nucleotide sequences of the ITS region.

Materials and Methods

Plant materials

Silica gel-dried leaf samples and voucher specimens of 15 *Weigela* taxa were collected in the field or botanic gardens. Voucher information for all the plant materials is listed in Table 14. Voucher specimens have been deposited in Makino Herbarium, Tokyo Metropolitan University.

Molecular analyses

Total genomic DNA was extracted from dried leaves using a cetyltrimethylammonium bromide method modified from Doyle and Doyle (1987). The ITS region of nuclear ribosomal DNA was amplified by PCR using a thermal cycler (GeneAmp PCR System 9700, Applied Biosystems, Foster City, USA). The following PCR primers were used: ITS1, which was used in previous study of *Weigela* (Kim & Kim 1999), and ITS4 (White et al. 1990). The ITS fragments were amplified using Nova Taq Hot Start DNA Polymerase (Novagen, Madison, WI), 1× Ampdirect Plus Buffer (Shimazu, Kyoto, Japan), and the primers of ITS1 and ITS4. The conditions for PCR amplifications were as follows: initial denaturation at 95°C for 10 min and 31 cycles at 95°C for 1 min, at 50°C for 1 min, and at 70°C for 1 min.

Finally, the PCR was terminated with a final extension for 10 min at 72°C. After confirmation of PCR amplification on a 1.0% agarose gel, the amplified products were incubated at 37°C for 30 min and 80°C for 20 min with ExoSAP-IT (USB, Cleveland, OH, USA) to remove any excess primers and nucleotides. For cycle sequencing reactions, a BigDye Terminator kit version 3.1 (Applied Biosystems, Foster City, CA, USA) was used. The nucleotide sequences were determined on an automated DNA sequencer (ABI PRISM 3100, Applied Biosystems, CA, USA).

Phylogenetic analysis

The genus *Diervilla* is considered to be sister to *Weigela* (Hara 1983) and was used as an outgroup in this study. To obtain more complete coverage of *Weigela* species, sequence data of the ITS region from four species of *Weigela* and three species of *Diervilla* were retrieved from a DNA database (NCBI) and included in analyses. Accession numbers of the retrieved sequences are shown in Table 14. The ITS sequences were aligned and analyzed separately by maximum parsimony (MP) and maximum likelihood (ML) analyses using MEGA version 6 (Tamura et al. 2013).

The MP tree was obtained using the Subtree–Pruning–Regrafting algorithm (Nei and Kumar 2000) with search level 1, in which initial trees were obtained by

random addition of sequences (10 replicates). Branch support was assessed by bootstrap analysis (Felsenstein 1985) with 1,000 replicates using the full heuristic search option.

The ML tree was obtained based on the Tamura–Nei model (Tamura 1993). An initial tree for the heuristic search was obtained automatically by applying neighbor joining and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood approach and then selecting the topology with highest log likelihood.

Results

Phylogenetic analyses

The total aligned length of the ITS was 611 characters. The MP analysis resulted in 10 most parsimonious trees (length = 47, CI = 0.93, RI = 0.97). The highest log likelihood was −1153.1652. Consensus trees from MP and ML analyses are shown in Figures 7 and 8, respectively. These trees are better resolved than the previous molecular tree reported by Kim and Kim (1999). *Weigela middendorffiana* and *W. maximowiczii* were revealed to be sister to the other species of the genus *Weigela*. Furthermore, the four species of the genus *Weigela* that change their flower colors after flowering (*W. coraeensis, W. decora, W. japonica,* and *W. subsessilis*) did not form a clade, but were scattered in several clades in the phylogenetic trees.

Discussion

Phylogenetic relationship between W. middendorffiana and W. maximowiczii

Based on the molecular phylogenetic trees (Figures 7 and 8), *W. middendorffiana* and *W. maximowiczii* were revealed to be sister clades of the other species of *Weigela*. Each of these two species was classified in a monomorphic section, *Calyptrostigma* or *Weigelastrum*, which were separated from the other sections by having several peculiar morphological characteristics including hairy and connivent anthers and yellow corolla. We accordingly concluded that *W. middendorffiana* and *W. maximowiczii* are distantly related to the other species of *Weigela*.

The phylogenetic positions of flower color-changing species

The four species of the genus *Weigela* that change their flower colors after flowering (*W. coraeensis, W. decora, W. japonica*, and *W. subsessilis*) did not form a clade but were scattered in several clades in the phylogenetic trees (Figures 7 and 8). The geographical distribution areas of these four species are largely different; *W. coraeensis* and *W. decora* are distributed mainly on the Pacific side of central Honshu, *W. japonica* in Kyushu, Japan (Hara 1983), and *W. subsessilis* in Korea (Chang 1997). These data suggest that the character of flower color change in *Weigela* arose

independently several times in each distribution area.

Phylogenetic relatedness in the molecular trees and the composition of the flower pigments

Weigela middendorffiana and *W. maximowiczii* were revealed to be sister of the other species of the genus, and the compositions of the flower pigments of these two species was also very different from those of the other species. Therefore, differences in the flower pigment composition were consistent with the phylogenetic relatedness indicated by the molecular phylogenetic analyses. Based on the fossil records, species of *Weigela* are considered to have expanded their distributions to East Asia, Europe, and the Arctic/Subarctic of North America during the Miocene, and had remained in these areas during the Pliocene (Ling et al. 2013). The distribution ranges of *W. maximowiczii* and *W. middendorffiana* are different from those of the other *Weigela* spp., where the former species are distributed in the cool areas of Hokkaido and northern Honshu, and the latters are not. Therefore, these two species might have received different natural selection pressures, such as under different pollinator fauna.

Table 14. *Weigela* and *Diervilla* taxa used in this study, their voucher information, and accession numbers of the ITS sequences.

Taxa	Collection sites and dates	Voucher	ITS
Weigela middendorffiana	Cultivated in Botanic Garden, Hokkaido University, Sapporo, Hokkaido, Japan, Jun 9,2010	SS030	
W. maximowiczii	Cultivated in Nikko Botanical Gardens, Graduate School of Science, The University of Tokyo, Tochigi pref., Japan, Jun 4,2010	SS032	
W. hortensis	Cultivated in Tsukuba Botanical Garden, National Museum of Nature and Science, Tsukuba, Ibaraki pref., Japan, May 11, 2009	SS001	
W. hortensis f.*albiflora*	Cultivated in Nikko Botanical Gardens, Graduate School of Science, The University of Tokyo, Tochigi pref., Japan, Jun 4,2010	SS004	
W. coraeensis	Ainohara, Atami, Shizuoka pref., Japan, May 20, 2010	SS037	
W. coraeensis var. *fragrans*	Mt. Mihara, Izu Oshima Island, Tokyo pref., Japan, August 6, 2011	SS008	
W. decora	Cultivated in Tsukuba Botanical Garden, National Museum of Nature and Sciense, Tsukuba, Ibaraki pref., Japan, May 11, 2009	SS016	
W. decora f.*unicolor*	Cultivated in Tsukuba Botanical Garden, National Museum of Nature and Sciense, Tsukuba, Ibaraki pref., Japan, May 11, 2009	SS011	
W. decora var.*amagiensis*	Cultivated in Botanical Gardens, Graduate School of Science, The University of Tokyo, Tokyo, Japan, Jun 3, 2010	SS012	
W. floribunda	Cultivated in Tsukuba Botanical Garden, National Museum of Nature and Sciense, Tsukuba, Ibaraki pref., Japan, May 11, 2009	SS022	

All the plant samples used and voucher specimens in this study were collected by S. Shimokawa.

Table 14. (continued)

Taxa	Collection sites and voucher information	Voucher	ITS
Weigela×*fujisanensis* f. *versicolor*	Cultivated in Nikko Botanical Gardens, Graduate School of Science, The University of Tokyo, Nikko, Tochigi pref., Japan, Jun 5, 2010	SS013	
W. amabilis	Cultivated in Tokyo Metropolitan Medicinal Plant Garden, Tokyo, Japan, Jun 29, 2010	SS040	
W. sanguinea	Cultivated in Tokyo Metropolitan Medicinal Plant Garden, Tokyo, Japan, Jun 29, 2010	SS042	
W. florida	Cultivated in Botanical Gardens, Graduate School of Science, The University of Tokyo, Tokyo, Japan, Jun 5, 2010	SS028	
W. japonica	Cultivated in Nikko Botanical Gardens, Graduate School of Science, The University of Tokyo, Tochigi pref., Japan, Jun 4,2010	SS025	
W. sinica	Kim & Kim (1999)		AF078715
W. praecox	Kim & Kim (1999)		AF078712
W. toensis	Kim & Kim (1999)		AF078710
W. subsessilis	Kim & Kim (1999)		AF078707
Diervilla lonicera	Kim & Kim (1999)		AF078722
D. sessilifolia	Kim & Kim (1999)		AF078723
D. ribularis	Kim & Kim (1999)		AF078721

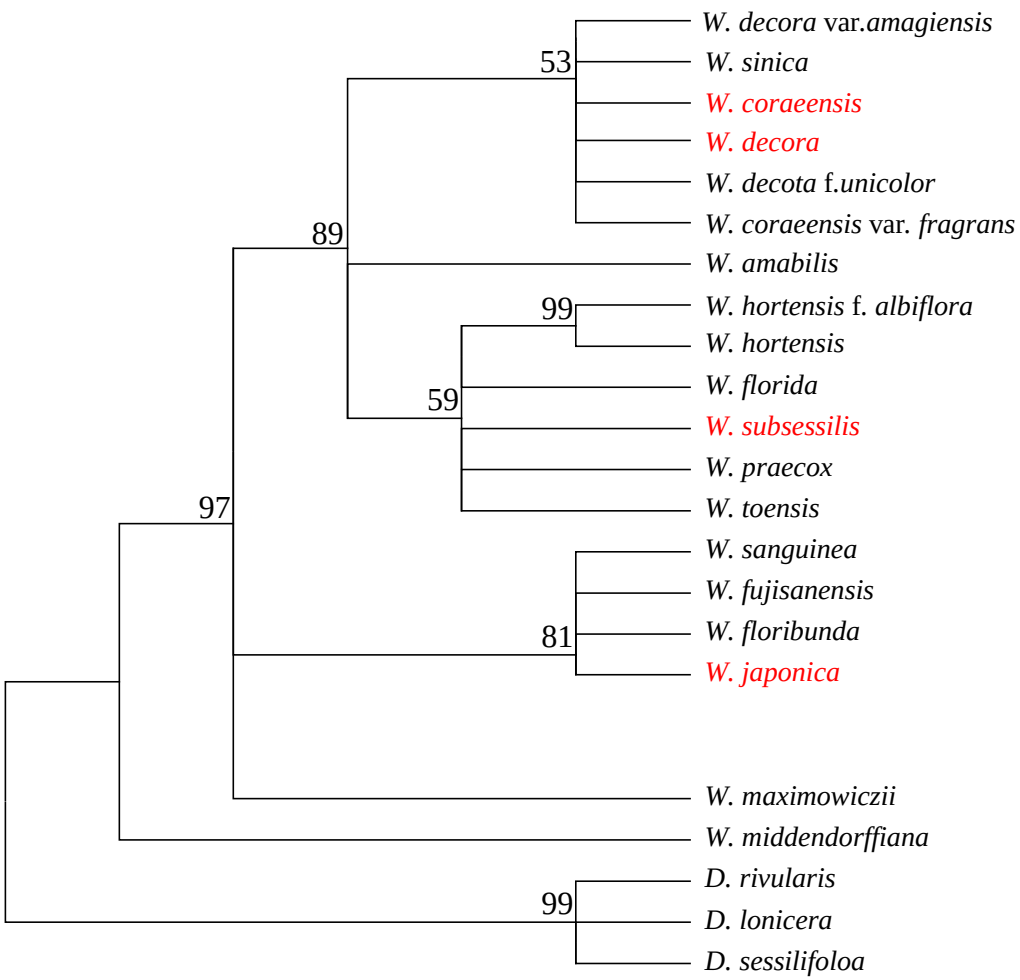


Figure 7. Consensus tree from maximum parsimony (MP) analyses based on ITS sequences from genera *Weigela* and *Diervilla*. Bootstrap probabilities greater than 50% are shown above branches. The names of the species that change flower colors after flowering are shown in red.

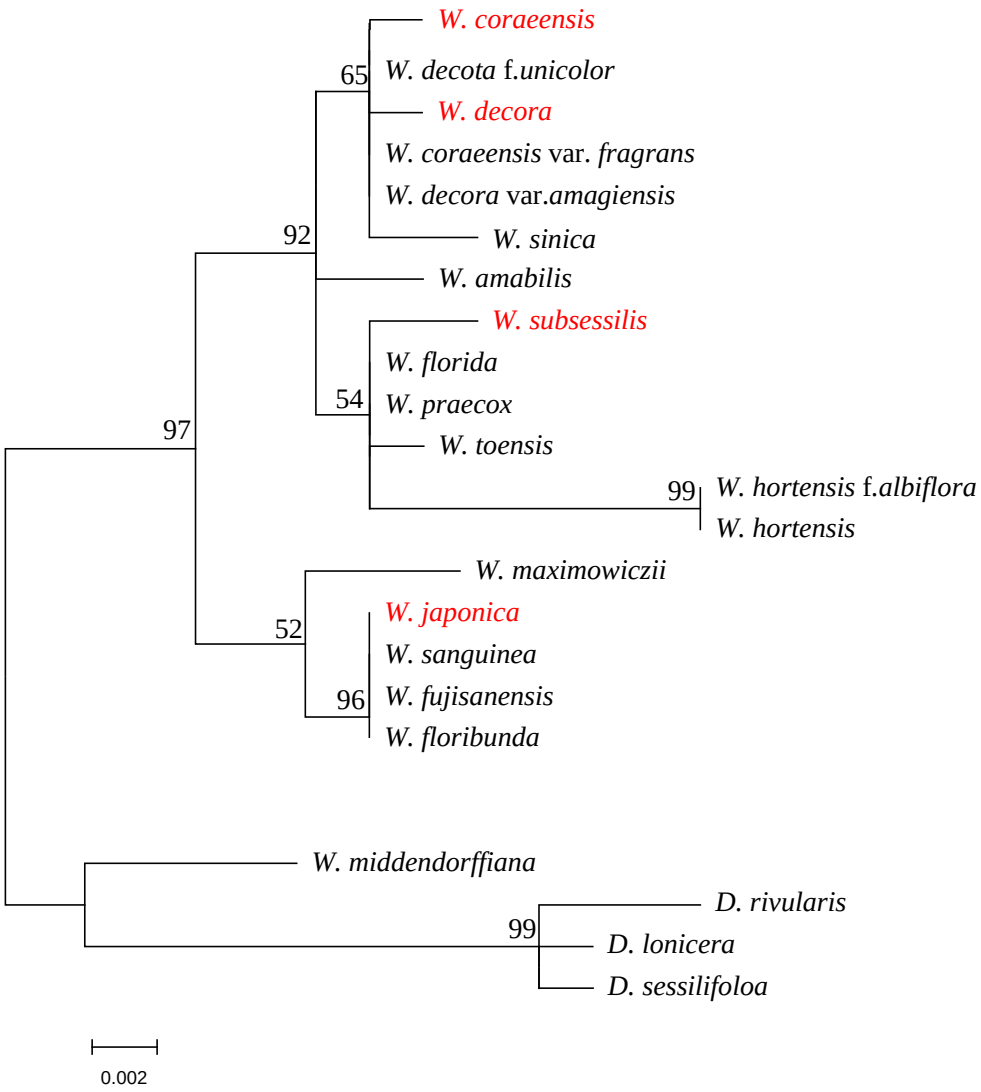


Figure 8. Consensus tree from maximum likelihood (ML) analyses based on ITS sequences from genera *Weigela* and *Diervilla*. Bootstrap probabilities greater than 50% are shown above branches. The tree is drawn to scale, with branch lengths corresponding to numbers of substitutions per site. The names of the species that change flower colors after flowering are shown in red.

Chapter III.

Effects of flower color change on pollinators of *Weigela coraeensis*

Introduction

Flower color change is a character occasionally observed in several angiosperm taxa across 33 orders, 76 families, and 268 genera (Weiss 1995). In these taxa, their flower colors change sequentially after flowering. Previous studies have shown that after a color change, flowers usually have less rewards (nectar and pollens) for their visitors and often lose both male and female reproductive capabilities (Gori 1983, Casper & La Pine 1984, Gori 1989, Oberrath & Böhning-Gaese 1999, Ida & Kudo 2003).

Several previous studies suggested that there are two effects of flower color change on pollinators. One effect is the enhanced attraction of the pollinators from long distances by plants that retain old flowers after a color change (Gori 1983, Casper & La Pine 1984, Cruzan et al. 1988, Delph & Lively 1989, Gori 1989, Weiss 1991, Niesenbaum et al. 1999, Oberrath & Böhning-Gaese 1999). Flower display depends on the number of opening flowers, and the plants with a larger flower display can often attract more pollinators (Cruzan et al. 1988, Klinkhamer et al. 1989, Klinkhamer & De

Jong 1990). The retention of old flowers without reproductive capabilities after a color change can contribute to maintaining the display size of the plants unlike the plants that lose their flowers. For example, *Aster vimineus* (Asteraceae) changes the center disk florets from yellow to red after flowering (Niesenbaum et al. 1999). In a previous study, the total number of pollinators arriving at a large *A. vimineus* patch (3.0 m^2), which consisted of yellow and red disk flowers, and a small *A. vimineus* patch (1.2 m^2), which consisted of only yellow disk flowers, were compared. As a result, more pollinators arrived at the larger flower display composed of a mix of yellow and red disk flowers than at the smaller flower display composed of only yellow disk flowers behind a smaller total number of flowers (Niesenbaum et al. 1999).

Another effect of flower color change is the guidance of pollinators to flowers with higher reproductive values at short distances (Casper & La Pine 1984, Gori 1989, Weiss 1991, Niesenbaum et al. 1999, Oberrath & Böhning-Gaese 1999). Most flower visitors, including diverse groups of insects and vertebrates can learn to associate color with a reward (Weiss & Lamont 1997, Willmer 2011). Thus, pollinators are expected to reduce visitation frequencies to flowers after a color change because these flowers tend to have a less amount of nectar reward. For example, *Cryptantha humilis* (Boraginaceae) changes the color of the corona (a small part of the corolla) of its

flowers from yellow to white after flowering (Casper & La Pine 1984). In this species, before the color change the flowers produce large amounts of nectar; however, after the color change they produce little nectar. It was reported that visitation frequency of pollinators to these flowers after color change was lower than that before the color change (Casper & La Pine 1984). Similarly, *Weigela middendorffiana* (Caprifoliaceae) changes the color of the nectar guide (a small part of the corolla) of its flowers from yellow to red after flowering. Younger yellow flowers offered about 10 fold more nectar than older red flowers in *W. middendorffiana*. As a result, the visitation frequency of pollinators to the flowers before the color change was reported to be higher than those after the color change (Ida & Kudo 2003).

In this study, we used *Weigela coraeensis*, which has flowers that sensationally change from white to red, and *Weigela coraeensis* f. *alba*, which has flowers that remain white even after flowering (Figure 9, 10). In other words, *W. coraeensis* has white and red bicolor flowers, whereas *W. coraeensis* f. *alba* has white monochrome flowers. Therefore, we hypothesize that the contrast shown by bicolor flowers should be more visually conspicuous than that shown by monochrome flowers even in the case of pollinators that are far away and that pollinators prefer to visit a plant with bicolor flowers than one with monochrome flowers. In addition, it has been reported that *W. coraeensis*

flowers produce large amounts of nectar before the color change and less amounts of nectar after the color change (Suzuki et al. 2014). In a previous study, pollinators recognized flower color change and they selectively visited flowers before the color change because the flowers had rewards in abundant quantities. Thus, it is expected that pollinators visit white flowers on the first day after flowering more frequently than they visit red flowers several days after flowering.

I have addressed the following questions in this study: (1) Who are the pollinators of *W. coraeensis* at my studying site? (2) Are the mating systems and floral characters the same between *W. coraeensis* and *W. coraeensis* f. *alba*? (3) Do the bicolor flowers of *W. coraeensis* attract pollinators more effectively than the monochrome flowers of *W. coraeensis* f. *alba*? (4) Do pollinators selectively visit flowers before a color change (white flowers) or after a color change (red flowers)?

Materials & Methods

Plant materials and study site

W. coraeensis is a deciduous shrub that grows to approximately 5 m in height and occurs in the coastal ranges. *W. coraeensis* f. *alba* is a less common form of *W. coraeensis* that comprises only white flowers. Both taxa flower from May to June and produce 5–15 bell-shaped flowers in corymbs. The length of the corolla tube is approximately 20–30 mm. *W. coraeensis* plants usually start to bloom when they are 50 cm tall and they typically have 10 to 20 flowers. When this species matures, it often has several thousands of flowers. The flower color of the whole corolla sensationally changes from white to red after flowering (Figure 9). During the color change on the second day after flowering anthocyanins in the corolla dramatically increase (Figure 4). Therefore, we considered flowers from the first day as white flowers, and flowers from the second day or later as red flowers. The color of the flowers of *W. coraeensis* f. *alba* remains white even after flowering (Figure 10).

This study was conducted in Ainohara-cho, Atami-shi, Shizuoka Prefecture, Japan (34°58'32"N, 138°22'58"E, elevation 320 m). I selected six plants of *W. coraeensis* and two of *W. coraeensis* f. *alba*, which were growing on sunny slopes or on the edge of a forest for my observations of flower visitors and experiments to determine the mating

system of these taxa.

The examination of the mating system

To investigate the mating system of *W. coraeensis*, I manipulated flowers on six randomly chosen plants from the middle of May to the beginning of June 2012. Each branch with several flower buds was used as a unit for manipulation and each of the following five pollination treatments were assigned to each branch: (1) a total of 101 unmanipulated flowers were naturally pollinated as a control; (2) 54 flowers were bagged and artificially self-pollinated to check for self-compatibility; (3) 55 flowers were bagged and artificially cross-pollinated to determine potential seed productivity using pollens for hand pollination collected from other individuals of the plant species; (4) 72 red flowers were bagged and artificially cross-pollinated using white flower pollens to determine the potential seed productivity of the red flower; and (5) 55 white flowers were bagged and artificially cross-pollinated using red flower pollens to determine the reproductive capability of these pollens from red flowers.

Pollen donors for hand pollination were selected from plants about 100 m apart from the manipulated individuals. Before flowering, each branch was enclosed in a 20 cm × 10 cm nylon mesh bag. In these experiments, I used the flowers on the first day

flowering as white flowers and on the fourth day flowering as red flowers. I collected the pollens from white flowers for pollination treatments (1) to (4) as well as the pollens from the red flowers for treatment (5). I harvested the mature fruits and counted the number of seeds and undeveloped ovules. Thus, I determined the fruit set (fruit/flower ratio) and seed set (seed/ovule ratio) for each of the manipulated flowers. To investigate whether fruit sets of each manipulated flowers were different, I used Fisher's exact test followed by Bonferroni method. To investigate whether seed sets of each manipulated flowers were different, I used the the Steel-Dwass's test.

The floral characteristics of W. coraeensis and W. coraeensis* f. *alba

I investigated fruit set by natural pollination, nectar production, and flower retention of *W. coraeensis* and *W. coraeensis* f. *alba* in May and June 2014. In these investigations, I manipulated the flowers from the four selected plants of *W. coraeensis* and two selected plants of *W. coraeensis* f. *alba*. Each branch with several flower buds was used as a unit for manipulation, and each investigation was assigned to each branch as follows.

First, I selected one plant in each taxa with nearly a thousand blooming flowers and observed the natural pollination of 80 and 50 unmanipulated flowers of *W.*

coraeensis and *W. coraeensis* f. *alba*, respectively. I harvested the mature fruits and determined the fruit set (fruit/flower ratio).

Second, nectar production in the flowers was examined for 63 and 38 flowers of *W. coraeensis* and *W. coraeensis* f. *alba*, respectively. An entire immature inflorescence of each branch was bagged with a 20 cm × 10 cm nylon mesh bag to exclude pollinators. After the flowers opened, nectar was obtained from the calyx tube using a glass microcapillary (Minicaps; Hirschmann Laborgeräte GmbH & Co. KG, Eberstadt, Germany) with a 1-ml volume. The volume was calculated on the basis of the length of the microcapillary filled with fluid.

Third, I examined the duration of flower retention for about 25 and 20 flowers of *W. coraeensis* and of *W. coraeensis* f. *alba*, respectively. All of the flowers on each branch were bagged with a 20 cm × 10 cm nylon mesh bag until they dropped without pollination. I counted the days from flowering to the falling of flowers.

The observation of flower visitors

I captured insect visitors to *W. coraeensis* for 15 days in total from 19th to 25th May, 2011 and 23rd to 31st May, 2012. I randomly chose three plants of *W. coraeensis* and then captured all the species of insects visiting the flowers. I killed all the captured

insects using ethyl acetate and identified these species. The sampling durations were 7 h for each day (9:00–16:00 in most cases).

I counted the number of all insects visiting the flowers of one of plant of *W. coraeensis*. The sampling durations were 7 h (9:00–16:00) for 9 days from 23rd to 31st May, 2012. I calculated the visiting frequency of each insect species to the flowers of *W. coraeensis*.

The determination of pollinators

I compared the fruit set and seed set after a single visit by each of the four kinds of insects (*Bombus ardens*, *Byasa alcinous*, *Ceratina japonica*, and *Lasioglossum* sp.) that most frequently visited the flowers at the study site (Table 15). I bagged an entire immature inflorescence with a 20 cm × 10 cm nylon mesh bag to exclude insect pollinators. I removed the bag when most of the flowers of the inflorescence had opened and then made it available for visitation by the four kinds of flower visitors. After a single visit by these insects, I tagged the plant and recorded the number of flowers visited and the total number of flowers in the inflorescence. The inflorescence was then bagged again and the ovaries were left intact until maturation. If the inflorescence was not visited by any insect during an observation period, it was bagged again and used in a

later observation.

I harvested mature fruits after ripening, and the number of seeds and undeveloped ovules were counted. I determined the fruit set (fruit/flower) and seed set (seed/ovule ratio) for each of the manipulated flowers. I considered mature seeds as signs of pollination success from a single visit by an insect. To investigate whether fruit sets after a single visit by each of the four kinds of insects were different, I used Fisher's exact test followed by Bonferroni method. To investigate whether seed sets after a single visit by each of the four kinds of insects were different, I used the the Steel-Dwass's test.

The constitution of pollinators of W. coraeensis and W. coraeensis* f. *alba

In this study, I selected one plant of *W. coraeensis* and one plant of *W. coraeensis* f. *alba* of similar size, which were only 8 m apart. I counted the number of visits by pollinators to the plants and compared the number between *W. coraeensis* and *W. coraeensis* f. *alba*. I counted one visit by a pollinator whether it left the plant or visited several flowers before leaving. The conspicuous flower did not bloom at least in 20 m of neighborhood except in these two plants. I removed some of the flowers so that the number of flowers in the two plants produced a similar display size (Table 16). Similarly, I removed some of the white flowers or some of the red flowers so that the

frequency of white and red flowers were the same in the *W. coraeensis* plants (Table 16). The observation durations were 7 h (9:00–16:00) for each of the 9 days from 26th to 29th May, 2013 and 23rd to 27th May, 2014. I compared the expected and actual numbers of visits to the plant by each insect species using the chi-square goodness-of-fit test.

The relative frequencies of visits to white and red flowers

I counted the number of the visits by pollinators to white flowers and red flowers of *W. coraeensis*. Moreover, I counted the number of flowers of each color on the plant every investigated day. Then I compared the number of pollinators that visited the white flowers and red flowers on the plants for each day of my observation. In this investigation, I selected one plant of *W. coraeensis* in 2011 and two plants of *W. coraeensis* in 2012. The observation durations were 7 h (9:00–16:00) for each of the 10 days from 21st to 24th May, 2011; 27th, 28th, and 31st May; and 4th, 5th, and 7th June, 2012.

The preference of pollinators for white flowers or red flowers was examined by comparing the expected number of visits to white flowers or red flowers within the plant and the actual number of visits to white flowers or red flowers on each observation

day. Furthermore, the expected number of visits to white flowers or red flowers within plants should depend on the relative frequency of the white flowers and red flowers within plants. The expected and actual number of visits to flowers of each color were compared using the chi-square goodness-of-fit test.

Results

The mating system of W. coraeensis

The proportion of fruit set was over 90% for the following pollination treatments: (1) unmanipulated naturally pollinated control, (3) artificially cross-pollinated, (4) bagged and red flowers artificially cross-pollinated using white flower pollens, and (5) bagged and white flowers artificially cross-pollinated using red flower pollens. The results of Fisher's exact test followed by Bonferroni method indicated that the fruit set of (2) bagged and artificially self-pollinated was significantly lower than those of the other treatments (Figure 11A). However, the differences among (1), (3), (4), and (5) were not significant (Figure 11A).

The seed sets for these treatments were 50–70%. However, the proportion of fruit set was 2% and the proportion of seed set was 3% for the pollination treatment (2) (Figure 11). The results of the Steel-Dwass's multiple comparison test indicated that the seed set of (2) was significantly lower than those of the other treatments (Figure 11B). The seed set of (5) was significantly lower than that of (1), (3), or (4), but significantly higher than that of (2) (Figure 11A). The differences among (1), (3), and (4) were not significant (Figure 11B).

The floral characteristics of W. coraeensis and W. coraeensis* f. *alba

The floral characteristics of *W. coraeensis* and *W. coraeensis* f. *alba* are shown in Table 17. The proportion of fruit set was 88% by natural pollination for both *W. coraeensis and W. coraeensis* f. *alba*. The mean nectar production and mean flower retention time for *W. coraeensis and W. coraeensis* f. *alba* were 2.40 μl and 4.36 days and 2.84 μl and 4.30 days, respectively.

Flower visitors and the frequencies of their visits

I identified 20 insect species visiting the flowers of *W. coraeensis*: ten species of bees (*Hymenoptera*), four species of hoverflies (*Diptera*), five species of butterflies (*Lepidoptera*), and one species of beetle (*Coleoptera*) (Table 15). I confirmed a few visits by the queen of *Bombus diversus* to the flowers of *W. coraeensis* in addition to the visits by the workers and males. Most of the flower visitors were foraging for nectar except for some hoverflies that were licking pollens directly from the stamens. All the species of bees accumulated corbicular pollen loads; except *Xylocopa appendicula circumvolans* that was a nectar robber.

The number of visits by each species of insects is shown in Table 15. The relative proportion of visits by each insect species within the total number of visits to the flowers

of *W. coraeensis* is shown in Figure 12. I could not distinguish *Lasioglossum* sp. from *Lasioglossum (Evylaeus)* sp. in a part of our observations, so I combined them into one category. However, the number of visits by *Lasioglossum sp*. exceeded that by *Lasioglossum (Evylaeus)* sp.. The number of the total visits by *B. ardens*, *C. japonica*, or *B. alcinous* were over 300. Similarly, the number of total visits by *Lasioglossum* sp. and *Lasioglossum (Evylaeus)* sp. were also over 300. Among them, the number of visits by *B. ardens* (including workers, queens, and males) was the largest comprising 86.4% of the total visits. The relative frequency of the visits by *C. japonica*, *B. alcinous*, and *Lasioglossum* sp., including *Lasioglossum (Evylaeus)* sp. were 6.9%, 3.8%, and 1.4% of the total visits, respectively.

The identification of effective pollinators

The proportion of fruit set after a single visit by *B. ardens* or *B. alcinous* was about 60% and as low as 5% for *C. japonica* or *Lasioglossum* sp. (Figure 13A). The proportion of seed set after a single visit by *B. ardens* or *B. alcinous* was about 70% and about 10% for *C. japonica* or *Lasioglossum* sp. (Figure 13B). The results of the Fisher's exact test followed by Bonferroni method indicated that the fruit set after a single visit by *B. ardens* or that by *B. alcinous* was significantly higher than that by *C. japonica* or

that by *Lasioglossum* sp. (Figure 13A). I found no significant difference between fruit set after a single visit by *B. ardens* and that by *B. alcinous,* and similarly, no significant difference between the fruit set after a single visit by *C. japonica* and that by *Lasioglossum* sp. (Figure 13A). The results of the Steel-Dwass's multiple comparison test indicated that seed sets after a single visit by the four insects were not significant (Figure 13B). Therefore, *B. ardens* and *B. alcinous* were identified as the main effective pollinators of *W. coraeensis* (Figure 14).

The preferences of the pollinators pertaining to W. coraeensis or W. coraeensis* f. *alba

The number of visits by workers and males of *B. ardens* to *W. coraeensis* and *W. coraeensis f. alba* are shown in Figure 15. I observed 80 and 478 visits by the workers of *B. ardens* to *W. coraeensis* and 34 and 340 visits by the workers of *B. ardens* to *W. coraeensis f. alba* in 2013 and 2014, respectively. In addition, I observed 197 visits by the males of *B. ardens* to *W. coraeensis* and 156 visits by the males of *B. ardens* to *W. coraeensis f. alba* in 2013. These differences between the numbers of visits to the two plant species were significant. In contrast, we also observed 230 visits by the males of *B. ardens* to *W. coraeensis* and 232 visits the males of *B. ardens* to *W. coraeensis f. alba* in

2014; however, the difference between the two plant species was not significant.

The preferences of the workers of B. ardens and B. alcinous pertaining to the white and red flowers

The visiting frequencies of *B. ardens* (workers) and *B. alcinous* to the white or red flowers of *W. coraeensis* are shown in Figure 16. The visiting frequencies of *B. ardens* (worker) to the white flowers were between 70% and 80% and those of *B. alcinous* were between 80% and 90% during the observed periods. Besides, the ratios of the white flowers in the plants were between 50% and 60%. The differences between either the visiting frequencies of *B. ardens* (workers) or *B. alcinous* to the white flowers and the ratios of the white flowers in the plant were significant for each day of my observation.

Discussion

The mating system of W. coraeensis and reproductive abilities of the red flowers

As shown in Figure 11, seed production by unmanipulated control flowers was almost the same as that by outcrossed flowers. This means that the amount of visitation by pollinators was enough for *W. coraeensis* at my study site. Self-pollinated flowers hardly produced seeds, indicating that *W. coraeensis* is self-incompatible.

These results also revealed that the pollen and stigma of the red flowers have reproductive capabilities. In a previous study, flower color change was usually accompanied by the loss of reproductive capability (Casper & La Pine 1984, Gori 1989, Niesenbaum et al. 1999, Ida & Kudo 2003). However, in *W. coraeensis*, even flowers that have changed their colors from white to red retained male or female reproductive capabilities.

The seed productivities of W. coraeensis and W. coraeensis f. *alba*

The proportions of fruit set to the total number of observed flowers under natural pollination for *W. coraeensis and W. coraeensis* f. *alba* were almost the same and were as high as about 90% (Table 17). This data indicates that the pollinators visited most flowers of both plants at the study site. Thus, the effect of flower color

change did not contribute to higher seed production, mean nectar production, or mean retention time of flowers because they were almost the same between *W. coraeensis* and *W. coraeensis* f. *alba* (Table 17). The data indicate that the flower characters changing pertaining to the two forms of the plant species were very similar except for their flower color change or retaining their original flower color. Thus, these two plant species were suitable for examining the role of flower color change.

The flower visitors and effective pollinators of W. coraeensis

I identified 20 insect species visiting the flowers of *W. coraeensis* with four species having much higher visitation frequencies than the others (Table 15, Figure 12). Therefore, I considered that these four species, which were *B. ardens*, *C. japonica*, *B. alcinous*, and *Lasioglossum* sp., could be the effective pollinators of *W. coraeensis* at the study site. Over 100 flowers were visited by *Xylocopa appendicula circumvolans*; however, this species robbed nectar from the outside of the flower tubes by piercing them near the base. For these reasons, I excluded the flower visitors other than the four main species from the candidates for the main pollinators of *W. coraeensis*.

Next, I compared fruit set and seed set after single visits by *B. ardens*, *B. alcinous*, *C. japonica*, or *Lasioglossum* sp. to determine the pollinator species of the plant. As a

result, seed production after pollination by *B. ardens* and *B. alcinous* were much higher than after pollination by *C. japonica* and *Lasioglossum* sp. (Figure 13). The latter two species of insects were remarkably smaller in body size and may not be able to touch stigmas and transfer pollens to them when they visited the flowers of *W. coraeensis*. It may be the reason why these two species were not effective pollinators. Accordingly, I concluded that *B. ardens* and *B. alcinous* were the main pollinators of *W. coraeensis* (Figure 14). Furthermore, *B. ardens* was observed to visit to the flowers at remarkably higher frequencies than *B. alcinous* (Figure 12). Hence, *B. ardens* should be the most important insect species contributing to the pollination of *W. coraeensis* at the study site.

The effect of flower color change on the pollinators over a long distance

In *Bombus ardens*, particularly the workers of the species preferred to visit the plant of *W. coraeensis* with both red and white flowers rather than those of *W. coraeensis f. alba* with only white flowers (Figure 15). Moreover, mean nectar production and mean retention time of flowers were almost the same between *W. coraeensis* and *W. coraeensis* f. *alba* (Table 17). Therefore, it can be concluded that the pollinators preferred to visit the plant with bicolor flowers than the plant with monochrome flowers. Bicolor flowers appeared more conspicuous to pollinators than monochrome flowers even from far away.

In other words, pollinators may be able to discover plants with bicolor flowers more easily than those with monochrome flowers from a distance. Similarly, plant species with bicolor fruits were reported to attract more birds than those with monochrome fruits (Willson & Thompson 1982, Willson & Melampy 1983). In these species, the unripe fruits have a different color and some of them have different colored accessory structures (e.g., bracts, peduncles, persistent calyces) as compared to the ripe fruits, where ripen and unripen fruits are in red and black, respectively.

Similar investigation has been conducted for *W. middendorffiana*, which changes flower color from yellow to red; however, it was reported that the number of visits by pollinators were not different between plants with bicolor flowers or those with monochrome flowers (Ida & Kudo 2010). There may be two reasons why my result pertaining to *W. coraeensis* differed from the previous research for *W. middendorffiana*. Primarily, the parts of the flowers that change color are different between the two plant species. In *W. coraeensis*, the color of the whole corolla changes, whereas that of *W. middendorffiana* changes only in the nectar guide part, which is a small part of the corolla. Thereby, the contrast in the flower colors of *W. middendorffiana* is much weaker than that in *W. coraeensis* and may be insufficient to attract the pollinators effectively. In addition, the number of flowers used in the previous study may not have been enough to detect the

enhanced attractions of the bicolor flowers. I used plants with more than 400 flowers for my investigation, whereas the previous research used plants with only 100 flowers. Consequently, the display size of the observed plants in the previous research may not be sufficient to attract the pollinators from far away.

The effect of flower color change on the pollinators over a short distance

My data shows that *B. ardens* (workers) and *B. alcinous* were the main pollinators of *W. coraeensis* and selectively visited the white flowers (Figure 16). It was previously reported that flower visitors remove most of the pollens on the first day after flowering and that nectar production decreases from the second day after flowering in *W. coraeensis* (Suzuki & Ohashi 2014). In other words, the red flowers have very little reward (nectar or pollens) for the pollinators. Besides, bumblebees and butterflies have already been shown to have the ability to associate color with nectar reward (Weiss 1997, Weiss & Papaj 2003, Kandori & Yamaki 2012). For these reasons, it can be supposed that *B. ardens* (worker) and *B. alcinous* can associate color with reward, which is abundant only in the white flowers and is in less amounts in the red flowers; furthermore, they have learned to selectively visit the white flowers.

Such a pollination behavior can be expected to affect pollination efficiency in

W. coraeensis. The white flowers had recently bloomed and were young, and it is more likely that they had not been pollinated as compared with the red flowers. In contrast, the red flowers had been flowering for several days and most of them should have been pollinated. Accordingly, these pollination behaviors that favor the white flowers increase the pollination efficiency in *W. coraeensis*.

The necessary condition that affects perception of flower color change by pollinators over long distances contributes to higher seed production

My investigation reveals that flower color change by *W. coraeensis* has a positive effect of attracting more pollinators from a distance than the monochrome flowers. Nevertheless, the proportions of fruit set under natural pollination for *W. coraeensis* and *W. coraeensis* f. *alba* were almost the same. The reason for the flower color change not contributing to the higher seed production by *W. coraeensis* may be due to the high number of pollinators at the study site. The pollinators preferred to visit *W. coraeensis* with bicolor flowers than to visit *W. coraeensis* f. *alba* with only white flowers. However, several hundred pollinators still visited the plants of *W. coraeensis* f. *alba* during the 4 or 5 days of my observation (Figure 15). Moreover, *B. ardens*, which was the most contributing pollinator for *W. coraeensis* at the study site, visited over 100

flowers during one visit to the plants of *W. coraeensis* and *W. coraeensis* f. *alba*. Therefore, the numbers of visits by the pollinators may be enough for most of the flowers of *W. coraeensis* f. *alba*. For these reasons, it can be assumed that flower color change will contribute to higher seed production when the pollinator visits are not enough i.e., when pollinators limit the seed production. I should perform the same investigation when the number of pollinators is small due to annual fluctuations.

Table 15. A list of flower visitors to *Weigela coraeensis* and the number of visits. Total numbers of their visits during my observation (6 days) as well as mean daily numbers of visits are shown.

Taxa	Flower visits	
	Total	Daily average
bees		
Bombus ardens	17706	2951.0
B. diversus	30	5.0
Tetraloniella nipponensis	5	0.8
Xylocopa appendicula circumvolans	117	19.5
Ceratina japonica	1408	234.7
Hylaeus nippon	5	0.8
Andrena sp.	4	0.7
Nomada sp.	1	0.2
Lasioglossum sp., *Lasioglossum (Evylaeus)* sp.	291	48.5
hoverflies		
Oligoneura nigroaenea	87	14.5
Episyrphus balteatus	41	6.8
Eristalis cerealis	1	0.2
Megaspis sp.	5	0.8
butterflies		
Byasa alcinous	787	131.2
Papilio bianor	63	10.5
P. memnon	14	2.3
Pieris rapae	1	0.2
Ochlodes ochraceus	6	1.0
beetle		
Cetonia roelofsi	37	6.2

Table 16. The number of (a) flowers on *Weigela coraeensis* and *Weigela coraeensis* f. *alba*, and (b) those of white and red flowers of *W. coraeensis* in each of the investigation days in 2013 and 2014.

Date (2013)	*W. coraeensis*	*W. coraeensis f. alba*
5 / 26	375	384
5 / 27	423	422
5 / 28	408	395
5 / 29	405	383
Date (2014)		
5 / 23	294	290
5 / 24	335	338
5 / 25	250	246
5 / 26	283	281
6 / 2	298	304

(a) The number of flowers of each color in *W. coraeensis*

Date (2013)	White flowers	Red flowers
5 / 26	187	188
5 / 27	181	242
5 / 28	180	228
5 / 29	218	187
Date (2014)		
5 / 23	165	129
5 / 24	174	161
5 / 25	134	116
5 / 26	130	153
6 / 2	158	140

Table 17. (a) Fruit set under open pollination, (b) mean nectar production per flower, and (c) mean duration of flower retention by *Weigela coraeensis* and *W. coraeensis* f. *alba*. Mean ± SE.

(a) Fruit set of open pollination (%/flower)

W. coraeensis	*W. coraeensis f. alba*
88 (80)	88 (50)

(b) Mean nectar production (μl/flower)

W. coraeensis	*W. coraeensis f. alba*
2.40 ± 0.14 (63)	2.84 ± 0.09 (38)

(c) Mean flower retention (days/flower)

W. coraeensis	*W. coraeensis f. alba*
4.36 ± 0.22 (25)	4.30 ± 0.23 (20)

(a)

(b)

Figure 9. The photograph of *Weigela coraeensis*. (a) An individual with bicolor flowers in white and red, (b) flower color change from white to red after flowering.

Figure 10. Photograph of *Weigela coraeensis* f. *alba* with monochrome flowers in white.

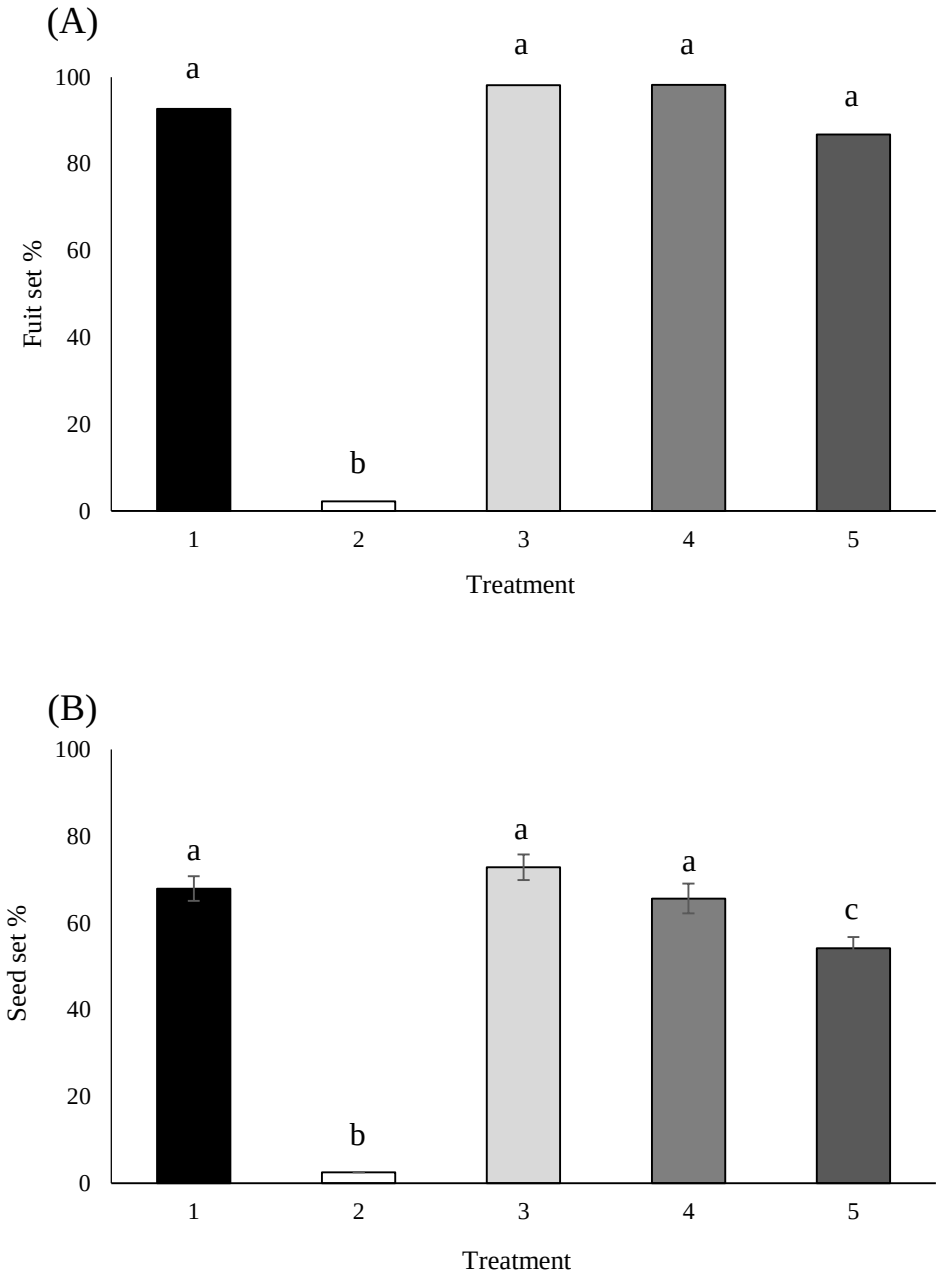


Figure 11. (A) Fruit set, and (B) seed set (mean ± SE) of *Weigela coraeensis* under (1) unmanipulated naturally pollinated control, (2) bagged and artificially self-pollinated, (3) bagged and artificially cross-pollinated, (4) bagged and red flowers artificially cross-pollinated using white flower pollens, (5) bagged and white flowers artificially cross-pollinated using red flower pollens. Means with the same letter do not differ significantly. (A), Fisher's exact test followed by Bonferroni method, $P > 0.05$; (B), the Steel-Dwass's multiple comparison test, $P > 0.001$.

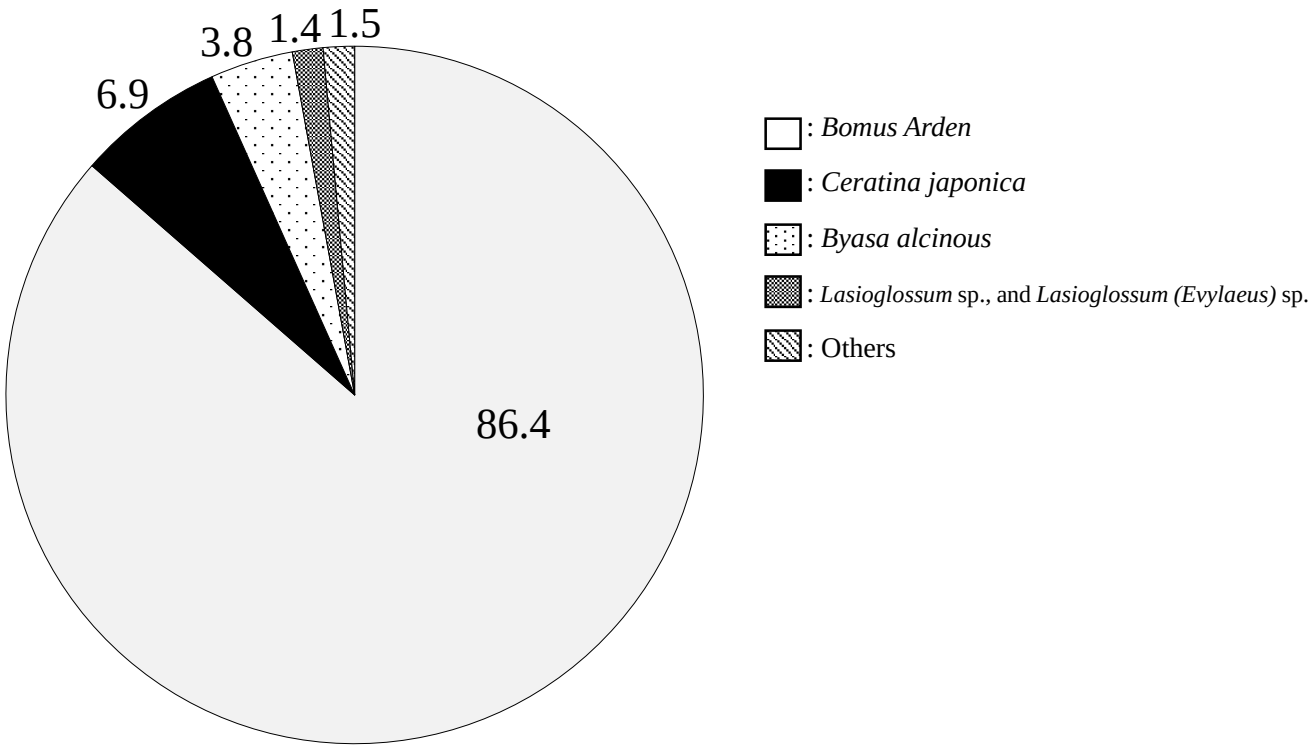


Figure 12. Relative frequency (%) of four kinds of insect of visitors in *Weigela coraeensis* flowers by the.

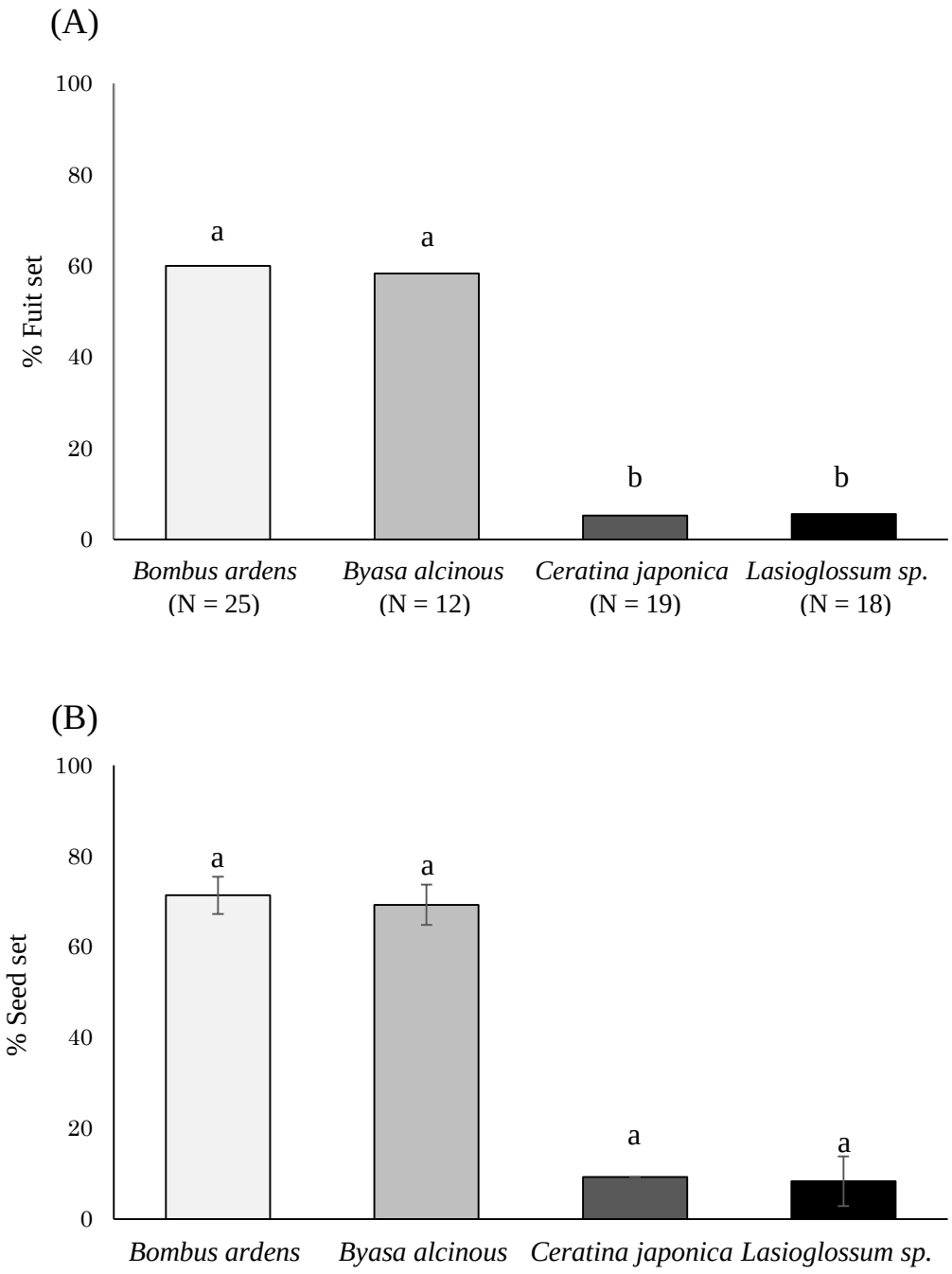


Figure 13. (A) Fruit set (mean), and (B) seed set (mean ± SE) after a single visitation by the four kinds of flower visitors. Means with the same letter do not differ significantly. (A), Fisher's exact test followed by Bonferroni methoed, $P > 0.05$; (B), the Steel-Dwass's multiple comparison test, $P > 0.05$.

Bombus ardens (worker)

(a)  (b) 

Bombus ardens (male)

(c)  (d) 

Byasa alcinous

(e)  (f) 

Figure 14. Photograph showing flower visitation by the pollinators of *W. coraeensis*.
(a) and (b), *Bombus ardens* (worker); (c) and (d), *B. ardens* (male); (d) and (f), *Byasa alcinous*.

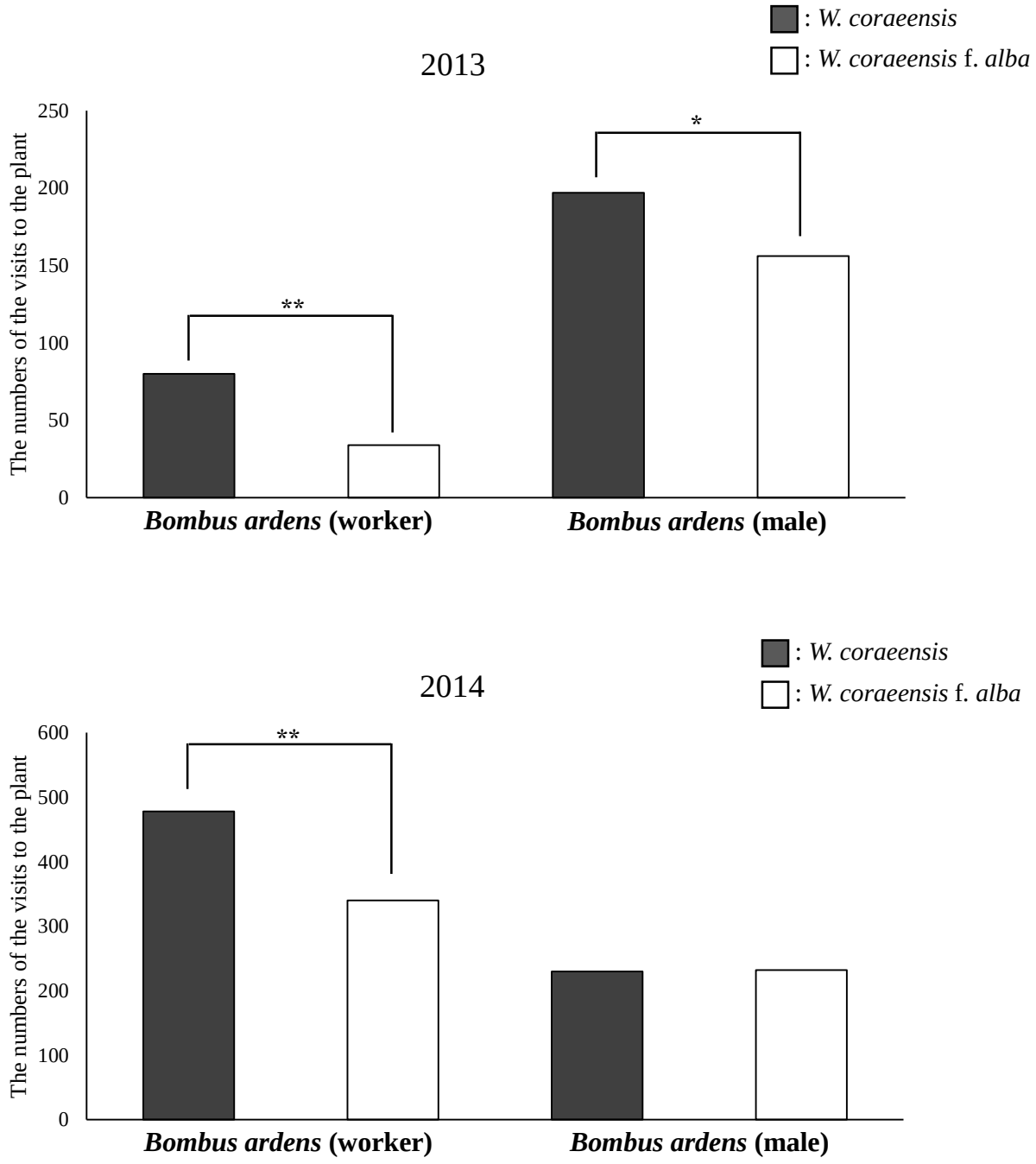
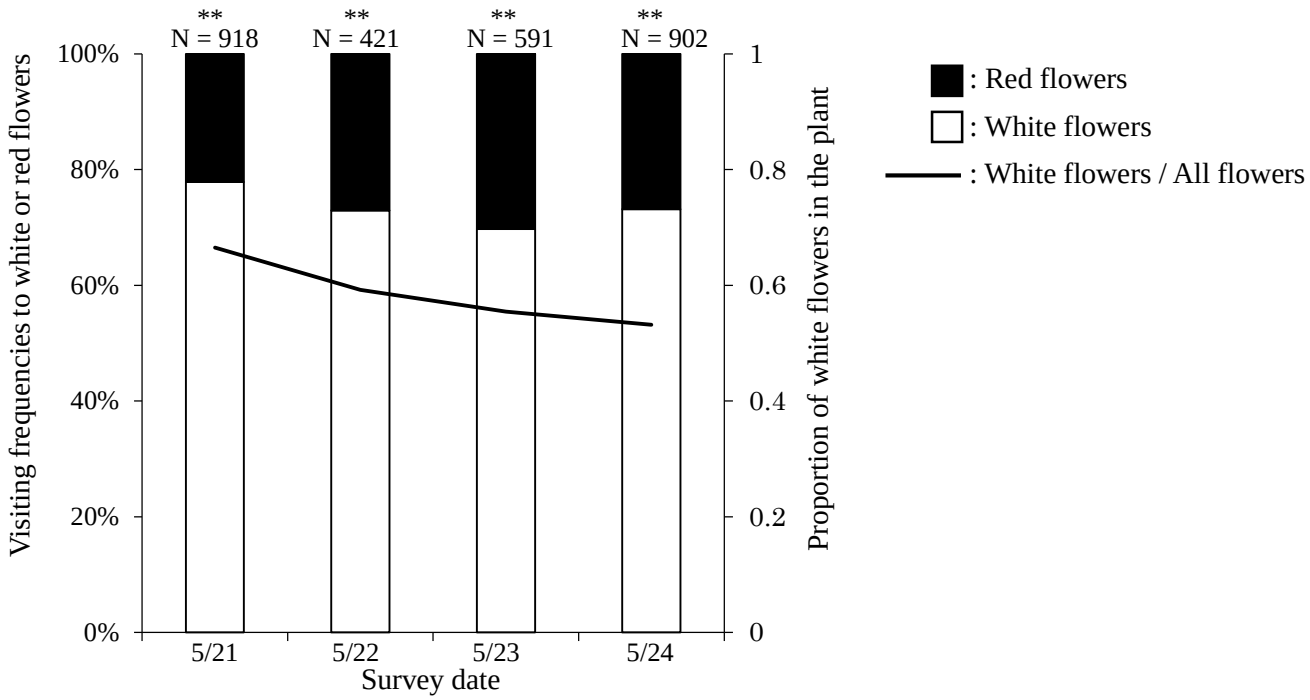


Figure 15. The number of visits by worker and male *Bombus ardens* to the plants of *Weigela coraeensis* and *Weigela coraeensis* f. *alba* in 2013 and 2014, respectively. ** $P < 0.001$ and * $P < 0.05$ using the chi-square goodness-of-fit test.

(a) *Bombus ardens* (worker)



(b) *Byasa alcinous*

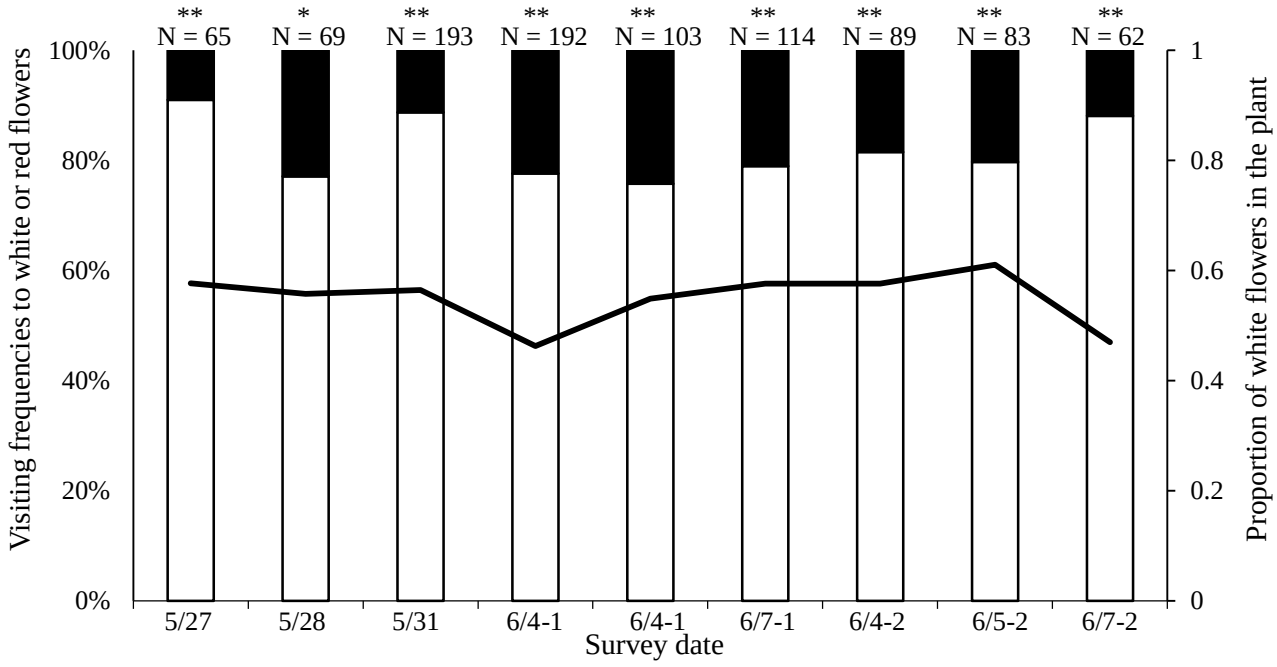


Figure 16. (a) The visiting frequencies of *Bombus ardens* (workers), and (b) *Byasa alcinous* to white flowers (on the first day from flowering) or red flowers (second day or later after flowering) of *Weigela coraeensis*. N indicates the number of flower visitations by the pollinators. ** $P < 0.001$ and * $P < 0.05$ using the chi-square goodness-of-fit test.

Chapter IV.

Flower color changes in three Japanese *Hibiscus* species: further characterization and quantitative variation of anthocyanin and flavonols

Introduction

Most flowering plants maintain their flower color after flowering, while some species change their colors sequentially after flowering. Plant taxa that change their flower colors are widespread across 268 genera and 76 families (Weiss 1995). Nevertheless, previous study of quantitative survey during the flower color change were rare. Therefore, biochemical mechanisms of the flower color changes were not well elucidated.

Hibisucus tiliaceus is a large shrub or tree species that is widely distributed in the Old Word tropics. In contrast, *H. hamabo* is distributed only in temperate coastal areas of Japan and Korea (Nakanishi 1985). These species grow in coastal thickets along brackish rivers (Kudoh et al. 1998; Nakanishi 1988). On the other hand, *H. glaber* is a endemic species of the Ogasawara Islands of Japan, and grows in inland dry scrubs (Shimizu 1992). It was suggested by phylogenetic analyses based on nucleotide sequence

data of chloroplast DNA (cpDNA) that *H. hamabo* and *H. glaber* were derived from *H. tiliaceus*, (Takayama et al. 2005).

Flower color changes of their *Hibiscus* species are general characters. Specifically, the flower colors of *H. tiliaceus* and *H. glaber* sensationally change from yellow to red in a day (Figure 17, 18), whereas that of *H. hamabo* changes from yellow to orange in two days (Figure 19). Regarding the biochemical mechanisms of flower color change in the petals of *Hibiscus*, it was reported that two anthocyanins, cyanidin 3-*O*-sambubioside and cyanidin 3-*O*-glucoside, were synthesized only in pink petals during the color change from white to pink of *H. mutabilis* var. *versicolor*. Thus, it was confirmed that the anthocyanins were produced during flower color change after flowering (Ishikura 1982; Amrhein & Frank 1989). However, other flavonoids are also contained in the petals of *H. mutabilis* var. *versicolor* (Subramanian & Swamy 1964). A quantitative survey of the pigments, including other flavonoids in *Hibiscus* flowers, has never been performed during the flower color change. In this chapter, I describe further characterization and the quantitative variation after flowering of flavonoids, including anthocyanins, in the petals of *H. hamabo*, *H. tiliaceus* and *H. glaber*.

Materials & Methods

Plant materials

Fresh petals of *Hibiscus hamabo* were collected near the mouth of the Oogamo River (Shimoda, Shizuoka, Japan) on July 22 to 25, 2013. Those of *H. tiliaceus* and *H. glaber* were collected from Chichijima Island, the Ogasawara Islands, Tokyo, Japan on October 20 to 26, 2013.

General

Preparative paper chromatography (PPC) and analytical cellulose TLC were performed using the following three solvent systems: BAW (*n*-BuOH/HOAc/H_2O = 4:1:5, upper phase), 15% HOAc and BEW (*n*-BuOH/EtOH/H_2O = 4:1:2.2). Preparative HPLC was performed on an *L*-column 2 ODS column (I.D. 10 × 250 mm, Chemicals Evaluation and Research Institute, Japan) at a flow-rate of 1.5 mL min^{-1}; detection, 350 nm (flavonols) and 530 nm (anthocyanin); eluting with MeCN/HOAc/H_2O/H_3PO_4 (4:8:85:3) for anthocyanins, and MeCN/H_2O/H_3PO_4 (20:80:0.2) for flavonols. UV spectra were recorded on a Shimadzu MPS-2000 multipurpose recording spectrophotometer (220-500 nm) according to Mabry et al. (1970). LC-MS was measured using an *L*-column 2 ODS column (I.D. 2.1×100 mm, Chemicals Evaluation

and Research Institute) at a flow-rate of 0.2 mL min^{-1}, eluting with MeCN:H_2O:HCOOH (6:89:5) for anthocyanins, and MeCN:H_2O:HCOOH (20:75:5) for flavonols, ESI^+ 4.5 kV, ESI^- 3.5 kV, 250°C. Isolated flavonoid glycosides were hydrolyzed with 12% HCl for 30 min on a boiling water bath. After cooling, the solution was shaken with diethyl ether, and the aglycones (diethyl ether phase) and sugars (mother liquor) were obtained.

Extraction and separation

Flower pigments were extracted from fresh petals (ca. 150 g) of *H. tiliaceus* with HCOOH/MeOH (8:92). After concentration, the extracts were fractionated by PPC using BAW, 15% HOAc and then BEW. The isolated flavonoids were purified by Sephadex LH-20 column chromatography using MeOH/HOAc/H_2O (70:5:25) for anthocyanins, and 70% MeOH for flavonols. The isolated flavonoids were further purified by preparative HPLC.

Identification

Flavonoids were identified by UV spectroscopy, LC-MS, acid hydrolysis, and HPLC comparisons with authentic samples.

Quantitative HPLC analysis

Five sets of fresh petals (each 0.2 g) of *H. hamabo* were collected from five different individuals of 0, 10, 20 and 30 hours after flowering. Those of *H. tiliaceus* and *H. glaber* (each 5 samples) were collected in 0, 2.5, 5, 7.5 and 10 hours after flowering. Anthocyanins and flavonols were extracted from the collected fresh petals with HCOOH/MeOH (8:92, 3 ml) and MeOH (3 ml), respectively. After filtration, the extracts were analysed by HPLC. Relative anthocyanin and flavonol contents were determined by the peak area of each compounds on the HPLC chromatograms.

Result

Identification of flavonoids

An anthocyanin and four flavonols were isolated from the petals of *Hibiscus hamabo, H. tiliaceus* and *H. glaber*. Cyanidin 3-*O*-sambubioside (**A4**) as anthocyanin, and gossypetin 3-*O*-glucuronide-8-*O*-glucoside (**F6**), quercetin 7-*O*-rutinoside (**F7**), gossypetin 3-*O*-glucoside (**F8**) and gossypetin 8-*O*-glucuronide (**F9**) as flavonols were identified by UV spectroscopy, LC-MS, acid hydrolysis, HPLC comparison with authentic samples. Each flavonoids were identified by the following data and Table 18 - 20.

Cyanidin 3-*O*-sambubioside (**A4**)

TLC (Rf): 0.13 (BAW), 0.63 (15% HOAc), 0.41 (BEW).

Color: Visible – red purple.

HPLC (Rt): 18.60 min.

UV λmax (nm): 0.01%HCl-MeOH| 280, 380sh, 531; +$AlCl_3$| 310, 359sh, 564

E_{acid}/E_{max}(%): 85, E_{440}/E_{max}(%): 24

LC-MS: *m/z* 581 $[M+H]^+$ (molecular ion peak, cyanidin + 1 mol xylose and glucose), *m/z* 419 $[M–162+H]^+$ (fragment ion peak, cyanidin + 1 mol xylose), *m/z* 287 $[M–294+H]^+$

(fragment ion peak, cyanidin).

Gossypetin 8-*O*-glucuronide 3-*O*-glucoside (**F6**)

TLC (Rf): 0.06 (BAW), 0.12 (15% HOAc), 0.30 (BEW).

Color: UV (365 nm) – dark purple, UV/NH_3 – yellow.

HPLC (Rt): 5.69 min.

UV λmax (nm): MeOH| 254, 272sh, 374; +NaOMe| 284, 437 (inc.); +$AlCl_3$| 258, 372; +$AlCl_3$/HCl| 247, 369, 428; +NaOAc| 280, 322, 410; +NaOAc/H_3BO_3| 254sh, 274, 331sh, 379.

LC-MS: *m/z* 657 $[M+H]^+$ (molecular ion peak, gossypetin + 1 mol glucuronic asid and glucose), *m/z* 495 $[M–162+H]^+$ (fragment ion peak, gossypetin + 1 mol glucuronic asid), *m/z* 319 $[M–338+H]^+$ (fragment ion peak, gossypetin).

Quercetin 7-*O*-rutinoside (**F7**)

TLC (Rf): 0.33 (BAW), 0.61 (15% HOAc), 0.42 (BEW).

Color: UV (365 nm) – dark purple, UV/NH_3 – yellow.

HPLC (Rt): 6.11 min.

UV λmax (nm): MeOH| 256, 372; +NaOMe| 240sh, 289, 373; +$AlCl_3$| 270, 450;

+$AlCl_3$/HCl| 266, 429; +NaOAc| 258, 394; +NaOAc/H_3BO_3| 260, 389.

LC-MS: *m/z* 611 $[M+H]^+$ (molecular ion peak, quercetin + 1 mol glucose and rhamnose), *m/z* 465 $[M–146+H]^+$ (fragment ion peak, quercetin + 1 mol glucose), *m/z* 303 $[M–308+H]^+$ (fragment ion peak, quercetin).

Gossypetin 3-*O*-glucoside (**F8**)

TLC (Rf): 0.17 (BAW), 0.03 (15% HOAc), 0.37 (BEW).

Color: UV (365 nm) – dark purple, UV/NH_3 – yellow.

HPLC (Rt): 8.68 min.

UV λmax (nm): MeOH| 252, 333sh, 379; +NaOMe| 285, 392 (inc.); +$AlCl_3$| 255sh, 282, 370sh, 443; +$AlCl_3$/HCl| 265, 367sh, 442; +NaOAc| 275, 377; +NaOAc/H_3BO_3| 262, 321.

LC-MS: *m/z* 481 $[M+H]^+$ (molecular ion peak, gossypetin + 1 mol glucose), *m/z* 319 $[M–162+H]^+$ (fragment ion peak, gossypetin).

Gossypetin 8-*O*-glucuronide (**F9**)

TLC (Rf): 0.14 (BAW), 0.04 (15% HOAc), 0.34 (BEW).

Color: UV (365 nm) – dark purple, UV/NH_3 – yellow.

HPLC (Rt): 12.96 min.

UV λmax (nm): MeOH| 260, 380; +NaOMe| 327, 437; +$AlCl_3$| 273, 444; +$AlCl_3$/HCl| 269, 440; +NaOAc| 280, 326sh, 403; +NaOAc/H_3BO_3| 264, 396.

LC-MS: *m/z* 495 $[M+H]^+$ (molecular ion peak, gossypetin + 1 mol glururonic asid), *m/z* 319 $[M–176+H]^+$ (fragment ion peak, gossypetin).

Distribution of anthocyanins and floavonols

Flower flavonoids of *H. hamabo*, *H. tiliaceus* and *H. glaber* were surveyed by HPLC. It was shown by qualitative HPLC analysis, flavonoid composition of *H. hamabo*, *H. tiliaceus* and *H. glaber* are very similar, especially the contents of the four flavonols (Table 21). However, the contents of cyanidin 3-*O*-sambubioside (**A4**) were different among three species. The petals of *H. hamabo* contained small amount of cyanidin 3-*O*-sambubioside (**A4**) (1.0%). On the other hand, those of *H. tiliaceus* and *H. glaber* contained large amounts of cyanidin 3-*O*-sambubioside (**A4**) (7.3% and 8.1%, respectively) (Table 21). Among the flavonoids detected in the petals, gossypetin 3-*O*-glucoside (**F8**) was major compound in *H. hamabo*, *H. tiliaceus* and *H. glaber* (70.9%, 73.5%, and 69.9%, respectively) (Table 21). Contents of the flavonols and anthocyanin increased after flowering. Especially, contents of the anthocyanin dramatically increased during the flower color change in *H. tiliaceus* and *H. glaber* (Figure 20).

Discussion

Identification of flavonoids

Cyanidin 3-*O*-sambubioside (**A4**) has already been reported from *Hibiscus mutabilis* f. *versicolor* and *H. sabdariffa* (Subramanian & Nair 1970, Pouget et al. 1990). As the anthocyanin of *Hibiscus* species, which were surveyed in this experiment, cyanidin 3-*O*-glucoside has been reported from the flowers of Malesian *H. tiliaceus* (Lowry 1976). However, this anthocyanin was not isolated from this species in this survey. Though flavonols, gossypetin 3-*O*-glucoside (**F8**) and gossypetin 8-*O*-glucuronide (**F9** have been reported from other *Hibiscus* species, *H. sabdariff*, and *H. vitiflorus* (Ismailov et al. 1994), respectively, gossypetin 3-*O*-glucuronide-8-*O*-glucoside (**F6**) and quercetin 7-*O*-rutinoside (**F7**) were reported from *Hibiscus* for the first time.

Distribution of anthocyanins and flavonols

In this study, flavonoid composition in the petals of *H. hamabo*, *H. tiliaceus* and *H. glaber* was shown to be exactly the same (Table 21), suggesting that these three species are closely related. Actually, it was reported that *H. hamabo*, *H. tiliaceus* and *H. glaber* formed a subclade in the most parsimonious trees based on nucleotide sequence data of cpDNA (Takayama et al. 2005). In addition, flavonoid contents of their species were

almost same except for cyanidin 3-*O*-sambubioside (**A4**). Cyanidin 3-*O*-sambubioside in the petal of *H. tiliaceus* and *H. glaber* was much higher than that of *H. hamabo* (Table 21). Flower colors of *H. tiliaceus* and *H. glaber* change from yellow to red, and that of *H. hamabo,* changes from yellow to orange, respectively. These difference of flower color change might be caused by the variation in the amount of cyanidin 3-*O*-sambubioside.

Furthermore, in *H. hamabo, H. tiliaceus* and *H. glaber*, ca. 80% of flavonoids in their petals were gosspetin glycoside (**F6**, **F8** and **F9**), especially gossypetin 3-*O*-glucoside (ca. 70%). I could not detect any carotenoid in the petals of *H. hamabo, H. tiliaceus* and *H. glaber* by UV spectral survey of their extracts. Almost all yellow flowers of the plants contain carotenoids, and these pigments often cause yellow color. However it is known that yellow flavonols, especially 6- and/or 8-substituted flavonols, can represent the deeper yellow color than common flavonols such as quercetin and kaempferol (Harborne 1969). Gossypetin is one of the yellow flavonol, and has been reported from other Malvaceous species, *Gossypium arboretum*, *G. hirsutum* and *G. barbadense*, as major pigments. Thus, it has been shown that the yellow flowers of these species are due to gossypetin (Parks 1965a, Parks 1965b, Parks 1967). In this survey, yellow color in the petal of *H. hamabo, H. tiliaceus* and *H. glaber* might be also due to gosspetin glycosides.

Quantitative changes of total amount of anthocyanins and flavonols

Flavonol and anthocyanin contents in *Hibiscus hamabo*, *H. tiliaceus* and *H. glaber* increased after flowering (Figure 20). These data indicate that flower color changes of *Hibiscus hamabo*, *H. tiliaceus* and *H. glaber* caused by increase of the flavonols and anthocyanin.

Table 18. TLC and HPLC data of anthocyanidins, which were obtained by acid hydrolysis of anthocyanins from the petals of *Hibiscus hamabo.*

anthocyanidins	Rf values			Colors	HPLC
	BAW	Forestal	BEW	(visible)	(Rt, min)
A4	0.67	0.40	0.46	light red purple	5.8
Authenic specimens:					
cyanidin	0.67	0.49	0.46	light red purple	5.8

BAW = *n*-BuOH/HOAc/H_2O (4:1:5, upper phase), 15%HOAc = HOAc/H_2O (15:85),
BEW = *n*-BuOH/HOAc/H_2O (4:1:2.2), Rt = retention time

Table 19. TLC and HPLC data of aglycones, which were obtained by acid hydrolysis of flavonols from the petals of *H. hamabo.*

Aglycones	Rf values			Colors		HPLC
	BAW	Forestal	BEW	UV	UV/NH3	(Rt, min)
F6	0.31	0.22	0.56	reddish brown	yellow	5.4
F7	0.82	0.41	0.88	reddish brown	yellow	9.2
F8	0.32	0.22	0.57	reddish brown	yellow	5.4
F9	0.31	0.23	0.57	reddish brown	yellow	5.4
Authenic specimens:						
gossypetin	0.32	0.23	0.57	reddish brown	yellow	5.4
quercetin	0.82	0.42	0.88	reddish brown	yellow	9.2

BAW = *n*-BuOH/HOAc/H_2O (4:1:5, upper phase), 15%HOAc = HOAc/H_2O (15:85),
BEW = *n*-BuOH/HOAc/H_2O (4:1:2.2), Rt = retention time.

Table 20. PC data of the glycosidic sugars, which were obtained by acid hydrolysis of anthocyanins and flavonols from the petals of *H. hamabo.*

Sugar	Rf values		Colors	Identity
	BBPW	BTPW		
A4	0.33	0.24	brownish yellow	glucose
	0.45	0.43	reddish brown	xylose
F6	0.33	0.29	brownish yellow	glucose
	0.06	0.05	red	glucuronic acid
	0.63	0.61	red	
F7	0.32	0.29	brownish yellow	glucose
	0.55	0.57	brownish yellow	rhamnose
F8	0.32	0.28	brownish yellow	glucose
F9	0.06	0.06	red	glucuronic
	0.62	0.61	red	acid
Authentic specimens:				
glucose	0.33	0.28	brownish yellow	
galactose	0.27	0.23	brownish yellow	
galacturonic acid	0.10	0.09	red	
glucuronic acid	0.06	0.06	red	
	0.63	0.61	red	
arabinose	0.37	0.36	reddish brown	
xylose	0.45	0.43	reddish brown	
rhamnose	0.59	0.57	brownish yellow	

BBTW = *n*-BuOH/Benzene/Pyridine/H_2O (5:1:3:3),

BTPW = *n*-BuOH/Toluene/Pyridine/H_2O (5:1:3:3).

Table 21. Distribution of flavonoids in the petals of *Hibiscus hamabo*, *H. tiliaceus* and *H. glaber*.

Compounds	Relative contents (%)		
	H. hamabo	*H. tiliaceus*	*H. glaber*
A4	1.0	7.3	8.1
F6	2.3	1.6	1.4
F7	2.6	1.0	1.4
F8	70.9	73.5	69.9
F9	5.0	5.3	5.5

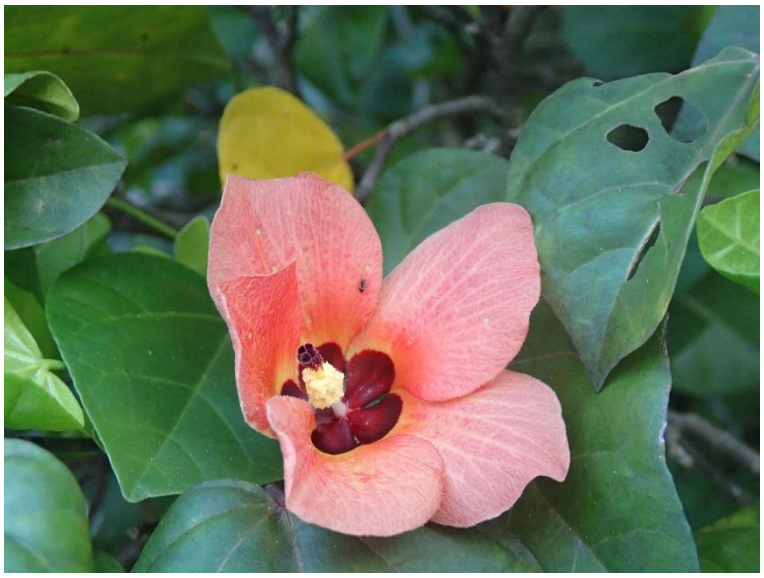
Petals of *H. hamabo* and *H. tiliaceus*, and *H. glaber* were extracted in 30 and 10 hours after flowering, respectively. **A4**, Cyanidin 3-*O*-sambubioside, **F6**, gossypetin 3-*O*-glucuronide-8-*O*-glucoside, **F7**, quercetin 7-*O*-rutinoside, **F8**, gossypetin 3-*O*-glucoside, and **F9**, gossypetin 8-*O*-glucuronide.

(a)

(b)

Figure 17. Photographs of *Hibiscus tiliaceus* showing (a) a flower before color change, and (b) a flower after color change.

(a)



(b)

Figure 18. Photographs of *H. glaber* showing (a) a flower before color change, and (b) a flower after color change.

(a)

(b)

Figure 19. Photographs of *H. hamabo* showing (a) a flower before color change, and (b) a flower after color change.

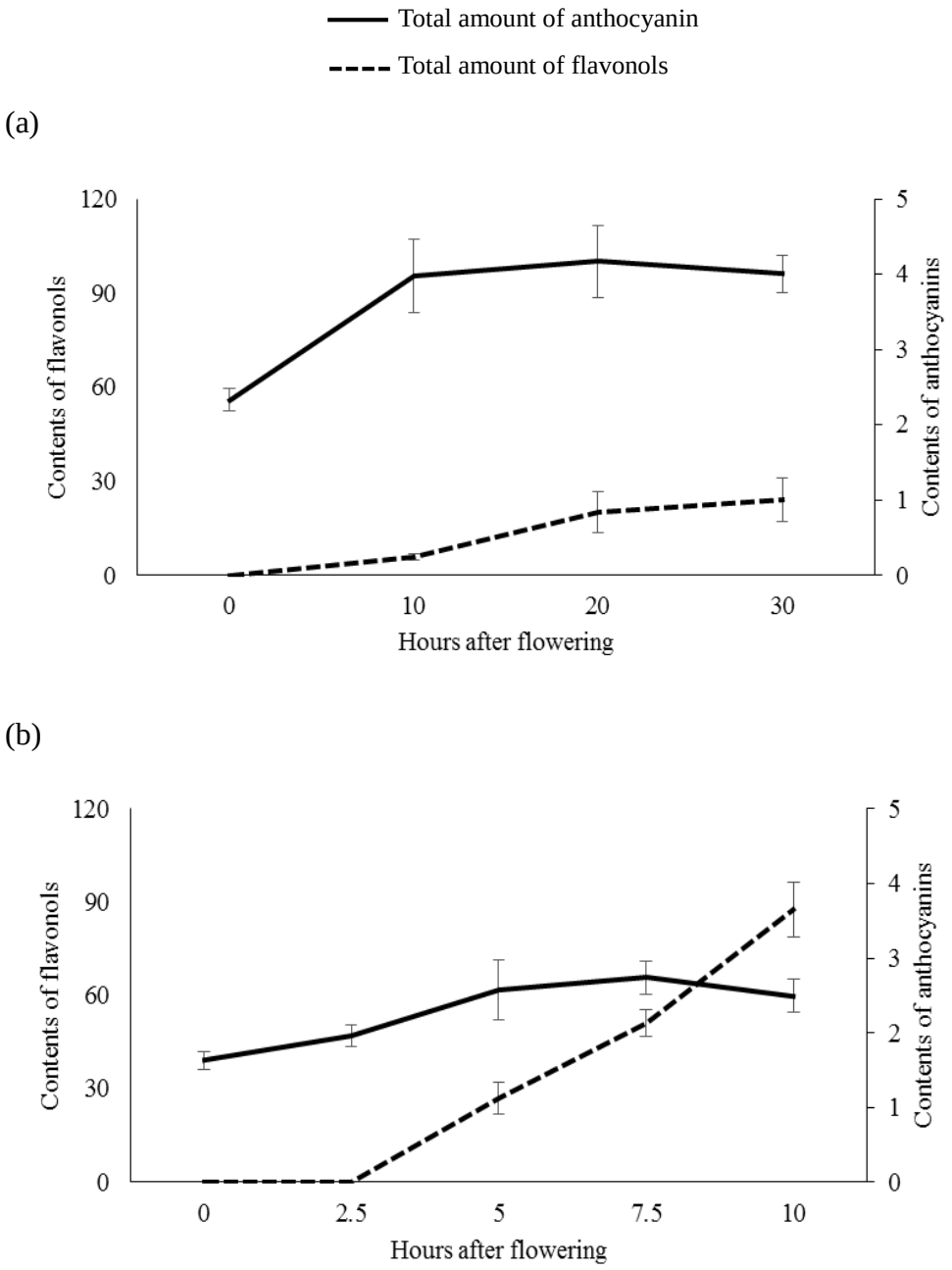


Figure 20. Quantitative changes of total amount of anthocyanin and flavonols in the petals of (a) *H. hamabo*, (b) *H. tiliaceus*, and (c) *H. glaber* (mean ± 1 SE). Left and right axes show the relative value. Relative amounts of the flavonoids are as 1.00 of peak area of the anthocyanin of 30 hours after flowering of *H. hamabo*.

(c)

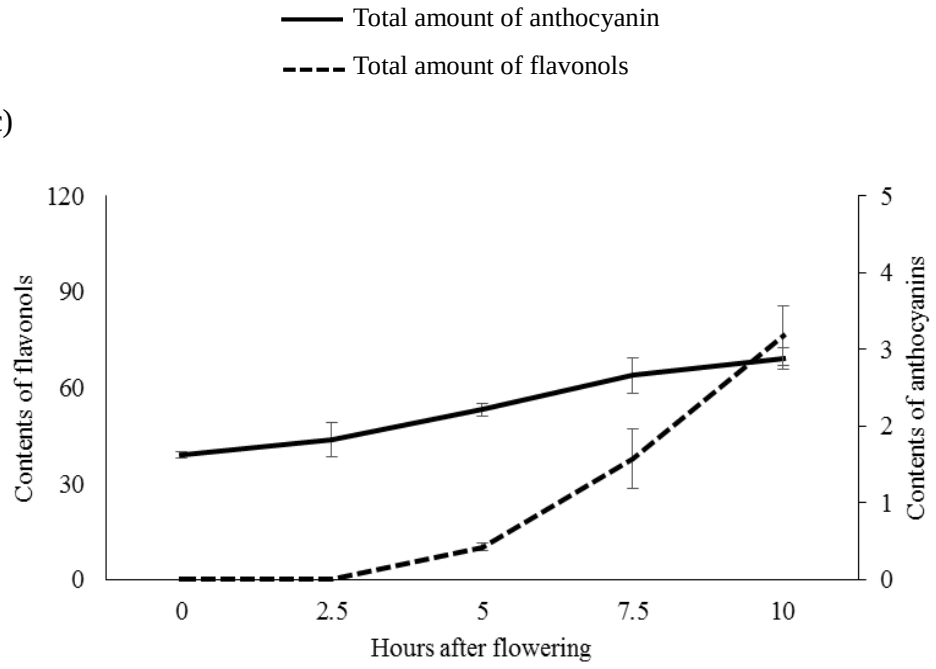


Figure 20. (continued) Quantitative changes of total amount of anthocyanin and flavonols in the petals of (a) *H. hamabo*, (b) *H. tiliaceus*, and (c) *H. glaber* (mean ± 1 SE). Left and right axes show the relative value. Relative amounts of the flavonoids are as 1.00 of peak area of the anthocyanin of 30 hours after flowering of *H. hamabo*.

General Discussion

Flower color change in plants evolved under interactions with their pollinators to more efficiently attract pollinators to the flowers (Chittka 1996). Flower color change is a characteristic, in which flower color changes after flowering, and it is observed in various angiosperm taxa (Weiss 1995). Previous studies have shown that flower color changes are caused by qualitative and quantitative changes in flavonoids, carotenoids, chlorophylls, and betareins (Weiss 1995, Farzad et al. 2002, Ishikura 1982, Amrhein & Frank 1989). Moreover, several studies have suggested that flower color change enhances the attraction of long-distance pollinators to the plants by retaining old flowers after the color change (Gori 1983, Gori 1989, Cruzan et al. 1988, Weiss 1991, Niesenbaum et al. 1999, Oberrath & Bo¨hning-Gaese 1999, Casper & La Pine 1984, Delph & Lively 1989). Another effect is guiding pollinators at short distances by changing the color of flowers with higher reproductive values (Casper & La Pine 1984, Gori 1989, Weiss 1991, Niesenbaum et al. 1999, Oberrath & Bo¨hning-Gaese 1999).

The purpose of this study was to clarify: (1) the biochemical mechanisms of the flower color changes observed in *Weigela* and *Hibiscus* spp. (chapter I, IV); (2) the phylogenetic relationships among *Weigela* spp. that do or do not change their flower color

were analyzed, based on the nucleotide sequences of the ITS region (chapter II); (3) the effects of flower color changes on pollinators in *W. coraeensis*, in which flower color changes from white to red after flowering (chapetr III).

Three anthocyanins and five flavonols were identified from *Weigela* corollas in Chapter I. In addition, the flower color changes in *Weigela* were determined to be caused by increases in anthocyanin content. Moreover, the compositions of the anthocyanins and flavonols contained in the corolla were very similar among most *Weigela* spp.; however, those of *W. maximowiczii* and *W. middendorffiana* were very different from the other species.

The four *Weigela* spp. that change flower color after flowering were scattered in several clades in the phylogenetic tree, based on the molecular phylogenetic analyses conducted in Chapter II. This finding suggests that the flower color change characteristic in *Weigela* was independently obtained several times in different areas of its distribution. In addition, *W. middendorffiana* and *W. maximowiczii*, which have peculiar yellow flowers, were revealed to be sister to the other *Weigela* spp. clade. Therefore, it was concluded that *W. middendorffiana* and *W. maximowiczii* are distantly related to the other *Weigela* spp.

The results of Chapter III indicated that *Bombus ardens*, particularly the workers

of this insect species, preferred to visit *W. coraeensis*, which has both red and white flowers, rather than *W. coraeensis f. alba* with only white flowers. Therefore, it can be concluded that the pollinators preferred to visit plants with bicolor flowers rather than those with monochrome flowers. In addition, the data show that *B. ardens* (workers) and *Byasa alcinous* (primary *W. coraeensis* pollinator) selectively visited white flowers. Consequently, such pollinator behavior is expected to affect pollination efficiency in *W. coraeensis*.

An anthocyanin and four flavonols were isolated and identified from the petals of *H. hamabo*, *H. tiliaceus*, and *H. glaber* in Chapter IV. Flavonol and anthocyanin contents in *H. hamabo*, *H. tiliaceus*, and *H. glaber* increased after flowering. This result indicates that flower color changes in these three *Hibiscus* spp. resulted from increases in flavonols and an anthocyanin.

Quantitative surveys of flower pigments during flower color changes have rarely been performed. Therefore, the biochemical mechanisms of flower color change are poorly understood. The results of this study revealed quantitative changes in the total amounts of anthocyanins and flavonols in *Weigela* and *Hibiscus*. Flower color changes in *Weigela* resulted from increases in the content of an anthocyanin, whereas the flower color changes in *H. hamabo*, *H. tiliaceus*, and *H. glaber* were caused by increases in both

flavonols and the anthocyanin. Additionally, typical floral characteristics, for example, flower retention times are very different between the two genera. The flower retention times of *Weigela* and *Hibiscus* are five to six days and one to two days, respectively. The characters of flower color change might have evolved in each growing environments or under interactions with different pollinators. *Weigela* species are distributed in temperate areas, whereas *Hibiscus* species are distributed mainly in tropical and subtropical areas. Thus, their pollinators should be very different. The differences in the biochemical mechanisms of flower color change might be caused by natural selection of pollinators.

Most flavonoids, including flavonol, are not recognized as color pigments by humans because humans can only detect light at wavelengths of 400–700 nm as color, whereas several flavonoids have absorption maxima at <400 nm. Thus, more species may change flower color in nature; however, they are not detected by humans. To solve this problem, it is necessary to further characterize quantitative variations in flavonoids after flowering in various plant species. It is well known that bees can recognize light as color at wavelengths of 300–600 nm (Peitsch et al. 1992, Briscoe & Chittka 2001). Moreover, some butterflies and birds can recognize light as color at wider ranges of 300–700 nm (Briscoe & Chittka 2001, Altshuler 2003, Koshitaka et al. 2008). Therefore, these pollinators detect several flavonoids as color pigments, which are unrecognized by

humans. The present results clearly show that flower color changes caused by increases in anthocyanin content affected pollinator behavior. Thus, pollinators visiting flowers should be affected by flower color changes caused by flavonoids that cannot be recognize as color pigments by humans.

W. middendorffiana and *W. maximowiczii* were revealed to be sister to the clade of the other species, and the composition of the pigments contained in the flowers of *W. maximowiczii* and *W. middendorffiana* was also very different from that of the other species. In addition, several peculiar morphological characteristics, such as hairy and connivent anthers, are observed in *W. maximowiczii* and *W. middendorffiana*. Therefore, the morphological characteristics and flower pigment composition were consistent with the phylogenetic relationships indicated by the molecular phylogenetic analyses. *Weigela* spp. expanded to East Asia, Europe, and the Arctic/Subarctic of North America during the Miocene and remained distributed in these areas in the Pliocene (Ling et al. 2013). The *W. maximowiczii* and *W. middendorffiana* areas of distribution were different from those of other *Weigela* spp. Therefore, these two groups may have undergone different natural selection pressures, such as differences in pollinator fauna. In fact, *W. maximowiczii* and *W. middendorffiana* are currently mainly distributed in the cool areas of Hokkaido and northern Honshu, whereas the other *Weigela* spp. are distributed mainly

in the warmer areas of central and south Honshu (Hara 1983). Fossil species of *Weigela* probably disappeared from Europe and North America during the Pleistocene era however, to date, several species have survived in Asia (Ling et al. 2013). The current areas of *W. maximowiczii* and *W. middendorffiana* distribution in Japan are adjacent to those of the other *Weigela* spp.; however, these two species may have experienced different natural selection pressures than their pollinator fauna, particularly when inhabiting the colder areas.

The biochemical mechanisms of the flower color changes in *Weigela* and *Hibiscus* were revealed by identifying their flower pigments and quantitative variations in the pigments after flowering. Data from this study suggest that the flower color change character in *Weigela* developed several times independently in each lineage, possibly in different geographic areas. Moreover, the results show that pollinators preferred to visit *W. coraeensis*, which contains both red and white flowers, rather than *W. coraeensis f. alba* with only white flowers. Therefore, the pollinators preferred to visit plants with bicolor flowers rather than those with monochrome flowers. In addition, the data show that the *W. coraeensis* pollinators selectively visited young white flowers rather than the older red flowers. Finally, the data support the hypothesis that a change in flower color increases pollination efficiency, which could be why many angiosperm species have

evolved the flower color change characteristic.

Acknowledgements

I wish to express my gratitude to Professor Noriaki Murakami (Makino Herbarium, Tokyo Metropolitan University) for his encouragements and critical evaluation of this thesis. I also wish to express my gratitude to Professor Tsukasa Iwashina (Department of Botany, National Museum of Nature and Science) for helpful comments on my study. I thank Dr. Akira Shimizu (Tokyo Metropolitan University) for identification of the insect and assistance in my field works. I thank Dr. Takashi Sugawara, Dr. Hidetoshi Kato, and Dr. Yoko Kakugawa (Tokyo Metropolitan University) for their valuable discussion and advice.

I am grateful to Tsukuba Botanical Garden, Botanical Gardens of University of Tokyo, Nikko Botanical Garden of University of Tokyo, Botanical Gardens of Hokkaido University, The Kochi Prefectural Makino Botanical Garden, and Tokyo Metropolitan Medicinal Plant Garden for their permission to collect plant materials.

I express my cordial thanks to Dr. Yoshinori Murai (National Museum of Nature and Science), Dr. Ayumi Uehara (Department of Chemistry, Keio University),

and Dr. Miki Suzuki (Tsukuba University) for their valuable suggestions and help during the course of this study.

I am also grateful to Dr. Takayuki Yamada (National Museum of Nature and Science), Mr. Takuya Yajima, Mr. Hiroaki Kurushima, Miss. Asami Tanishima, Mr. Ryuto Nitta, Mr. Ken Matsumura, Mr. Yuya Tanabe, Mr. Yusuke Hoshino, Miss. Rena Nakajima (Tokyo Metropolitan University) for their kind supports of my field works.

Finally, I would like to thank all the members of Makino Herbarium of Tokyo Metropolitan University and Laboratory of Plant Chemotaxonomy of National Museum of Nature and Science for their encouragements and generous supports.

Literature Cited

Altshuler, D. L. 2003. Flower color, hummingbird pollination, and habitat irradiance in four neotropical forests. Biotropica 35: 344-355.

Amrhein, N. and G. Frank. 1989. Anthocyanin formation in the petals of *Hibiscus mutabilis* L. Z. Naturforsch. 44: 357-360.

Anderson, Ø. M. and K.R. Markham. 2006. Flavonoids: Chemistry, Biochemistry and Applications. CRC Press. Boca Roton.

Briscoe, A. D. and L. Chittka. 2001. The evolution of color vision in insects. Annu. Rev. Entomol. 46: 471-510.

Casper, B. B. and T. R. La Pine. 1984. Changes in corolla color and other floral characteristics in *Cryptantha humilis* (Boraginaceae): Cues to discourage pollinators? Evolution 38: 128-141.

Chang, C. S. 1997. Flavonoid chemistry of *Weigela* (Caprifoliaceae) in Korea. J. Plant Res. 110: 275-281.

Chittka, L. 1996. Does bee color vision predate the evolution of flower color? Naturwissenschaften 83: 136-138.

Cruzan, M. B., P. R. Neal and M. F. Willsn. 1988. Floral display in *Phyla incisa*: consequences for male and female reproductive success. Evolution 42: 505–515.

Delph, L. F. and C. M. Lively. 1989. The evolution of floral color change: pollinator attraction versus physiological constraints in Fuchsia excorticata. Evolution 1252-1262.

Doyle, J. J. and J. L. Doyle. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf material. Phytochem. Bull. 19: 11-15.

Faegri, K. L. and van der Pijl. 1966. The Principles of Pollination Ecology. Pergamon. Oxford.

Farzad, M., R. Griesbach and MR. Weiss. 2002. Floral color change in *Viola cornuta*L. (Violaceae): a model system to study regulation of anthocyanin production. Plant Sci. 162: 225-231.

Felsenstein, J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39:783-791.

Fenster, C. B., W. S. Armbruster, P. Wilson, M. R. Dudash and J. D. Thomson. 2004. Pollination syndromes and floral specialization. Annu. Rev. Ecol. Evol. Syst. 35: 375-403.

Gori, D. F. 1983. Post-pollination phenomena and adaptive floral changes. In Jones, C. E. and R. J. Little, Handbook of Experimental Pollination Biology. Van Nostrand Reinhold. New York. pp. 31-49.

Gori, D. F. 1989. Floral color change in *Lupinus argenteus* (Fabaceae): why should plants advertise the location of unrewarding flowers to pollinators? Evolution 43: 870-881.

Hara, H. 1983. Revision of Caprifoliaceae of Japan. Ginkgoana 5: 136-169.

Harborne JB. 1969. Gossypetin and herbacetin as taxonomic markers in higher plants. Phytochemistry 8: 177-183.

Hattori, S. and K. Hayashi. 1937. Studien über Anthocyane, II. Über die Farbstoffe aus den rotten Herbstblättern von einigen Acer-Arten. Acta Phytochim. 10: 129-138.

Hayashi, K. 1939. Studien über Anthocyane, V. Über die Farbstoffe den Beeren von *Fatsia japonica*. Acta Phytochim. 11: 91-108.

Ida, T. Y. and G. Kudo. 2003. Floral color change in *Weigela middendorffiana* (Caprifoliaceae): reduction of geitonogamous pollination by bumble bees. Am. J. Bot. 90: 1751-1757.

Ida, T. Y. and G. Kudo. 2010. Modification of bumblebee behavior by floral color change and implications for pollen transfer in *Weigela middendorffiana*. Evol Ecol. 24: 671-684.

Ishikura, N. 1982. Flavonol glycosides in the flowers of *Hibiscus mutabilis* f. *oversicolor*. Agr. Bio. Chem. 46: 1705-1706.

Ismailov, A. I., A. K. Karimdzhanov, Sh. Yu. lslambekov and Z. B. Rakhimkhanov. 1994. Flavonoids of the cotton plant and plants close to it. Chem. Nat. Compd. 30: 1-14.

Iwashina, T. and H. Hatta. 1994. The flavonoid glycosdes in the leaves of *Cornus* species IV. The distribution of flavonoids in the genus *Cornus*. Ann. Tsukuba Bot. Gard. 13: 29-40.

Iwashina, T. and J. Kitajima. 2009. Flavonoids from the leaves of betalain-containing species, *Phytolacca americana* (Phytolaccaceae). Bull. Natl. Mus. Nature Sci. Ser. B. 35: 99-104.

Iwashina, T., J. A. López-Sáez and J. Kitajima. 2008. *Flavonoids from Osyris alba*. Biochem. Syst. Ecol. 36: 146-147.

Iwashina, T., S. Ootani, T. Gotoh and N. Kondo. 1982. Distribution of flavonol glycosides in the sub-family Cereoideae and some chemotaxonomic consideration thereof. Sci. Rep. Res. Inst. Evol. Biol. 1: 83-102

Iwatsuki, K., T. Yamazaki, D. E. Boufford and H. Ohba. 1993. *Flora of Japan*, vol. 3a, *Angiospermae Dicotyledonae sympetalae*. Kokansha. Tokyo.

Kandori, I. and T. Yamaki. 2012. Reward and non-reward learning of flower colours in the butterfly *Byasa alcinous* (Lepidoptera: Papilionidae). Naturwissenschaften

99: 705-713.

Kim, Y. D. and S. H. Kim. 1999. Phylogeny of *Weigela* and *Diervilla* (Caprifoliaceae) based on nuclear rDNA ITS sequences: biogeographic and taxonomic implications. J. Plant Res. 112: 331-341.

Klinkhamer, P. G and T. J. de Jong. 1990. Effects of plant size, plant density and sex differential nectar reward on pollinator visitation in the protandrous *Echium vulgare* (Boraginaceae). Oikos 399-405.

Klinkhamer, P. G., T. J. de Jong and G. J. de Bruyn. 1989. Plant size and pollinator visitation in Cynoglossum officinale. Oikos 201-204.

Koshitaka, H., M. Kinoshita, M. Vorobyev and K. Arikawa. 2008. Tetrachromacy in a butterfly that has eight varieties of spectral receptors. Proc. Biol. Sci. 275: 947-954.

Kudoh, H., M. Uchiyama and N. Kachi. 1998. Flower size variation in *Hibiscus glaber* and *Hibiscus tiliaceus* in Chichijima Island, the Bonin (Ogasawara) Islands. Ogasawara Res. 24: 25-34.

Liang, X. Q., Y. Li., Z. Kvaček., V. Wilde and C. S. Li. 2013. Seeds of *Weigela* (Caprifoliaceae) from the Early Miocene of Weichang, China and the biogeographical history of the genus. Taxon 62: 1009-1018.

Lowry, J. B. 1976. Floral anthocyanins of some Malesian *Hibiscus* species. Phytochemistry 15: 1395-1396.

Mabry, T. J., K. R. Markham and M. B. Thomas. 1970. The Systematic Identification of Flavonoids. Springer. Berlin.

Muthusamy, A. and N. Jayabalan. 2011. Induced floral variations in cotton (*Gossypium hirsutum L.*). Plant Mutat Rep. 2. 12-17.

Nakanishi, H. 1985. Geobotanical and ecological studies on three semi-mangrove plants in Japan. Jpn. J. Ecol. 35: 85-92.

Nakanishi, H. 1988. Dispersal ecology of the maritime plants in the Ryukyu Islands, Japan. Ecol. Res. 3: 163-173.

Nei, M. and S. Kumar. 2000. Molecular Evolution and Phylogenetics. Oxford University Press. New York.

Niesenbaum, R. A., M. G. Patselas. and S. D. Weiner. 1999. Does flower color change in *Aster vimineus* cue pollinators? Am. Midland Nat. 141: 59-68.

Oberrath, R. and K. Böhnig-Gaese. 1999. Floral color change and the attraction of insect pollinators in lungwort (*Pulmonaria collina*). Oecologia 121: 383-391.

Parks, C. R. 1965a. Floral pigmentation studies in the genus *Gossypium* I. Species specific pigmentation patterns. Am. J. Bot. 52: 309-316.

Parks, C. R. 1965b. Floral pigmentation studies in the genus *Gossypium* II. Chemotaxonomic analysis of diploid *Gossypium* species. Am. J. Bot. 52: 849-856.

Parks, C. R. 1967. Floral pigmentation studies in the genus *Gossypium* III. Qualitative analysis of total flavonol content for taxonomic studies. Am. J. Bot. 54: 306-315.

Peitsch, D., A. Fietz., H. Hertel., J. Souza., D. F. Ventura. and R. Menzel. 1992. The spectral input systems of hymenopteran insects and their receptor-based colour vision. J. Comp. Physiol. A 170: 2340.

Pouget, M. P., B. Vennat, B. Lejeune. and A. Pourrat. 1990. Identification of anthocyanins of *Hibiscus sabdariffa* L. Leben. Wiss. Tech. 23: 101-102.

Shimizu, Y. 1992. Origin of *Distylium* dry forest and occurrence of endangered species in the Bonin Islands. Pacific Sci. 46: 179-196.

Subramanian, S. S. and A. G. R. Nair. 1970. A note on the color change of the flowers of *Hibiscus mutabilis*. Curr. Sci. 39: 323-234.

Subramanian, S. S. and M. N. Swamy. 1964. Flavonoids of the flowers of *Hibiscus mutabilis*. Curr. Sci. 33: 112-113.

Suzuki, M. F. and Ohashi, K. 2014. How does a floral colour‐changing species differ from its non‐colour‐changing congener?–a comparison of trait combinations and their effects on pollination. Funct. Ecol. 28: 549-560.

Takayama, K., T. Ohi-Toma., H. Kudoh. and H. Kato. 2005. Origin and diversification of *Hibiscus glaber*, species endemic to the oceanic Bonin Islands, revealed by chloroplast DNA polymorphism. Mol. Ecol. 14: 1059-1071.

Tamura, K. and M. Nei. 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol. Biol. Evol. 10:512-526.

Tamura, K., G. Stecher., D. Peterson., A. Filipski. and S. Kumar. 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol. Biol. Evol. 30: 2725-2729.

Weiss, M. R. 1991. Floral color change as cues for pollinators. Nature. 354: 227–229.

Weiss, M. R. 1995. Floral color change: a widespread functional convergence. Amer. J. Bot. 82: 167-185.

Weiss, M. R. 1997. Innate colour preferences and flexible colour learning in the pipevine swallowtail. Anim. Behav. 53: 1043-1052.

Weiss, M. R. and B. B. Lamont. 1997. Floral color change and insect pollination: a dynamic relationship. Isr. J. Plant Sci. 45(2-3). 185-199.

Weiss, M. R. and D. R. Papaj. 2003. Colour learning in two behavioural contexts: how much can a butterfly keep in mind? Anim. Behav. 65: 425-434.

Weiss, M. R. and B. B. Lamont. 1997. Floral color change and insect pollination: a dynamic relationship. Isr. J. Plant Sci. 45: 185-199.

White, T. J., T. Birns, S. Lee and J. Taylor. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *In*: Innis, M., D. Gelfand, J. Sninsky and T. White, PCR Protocols: A Guide to Methods and Applications. Academic Press. San Diego. pp. 315-322.

Willmer, P. 2011. *Pollination and floral ecology*. Princeton University Press. New York.

Willson, M. F. and M. N. Melampy. 1983. The effect of bicolored fruit displays on fruit removal by avian frugivores. Oikos 41: 27-31.

Willson, M. F. and J. N. Thompson. 1982. Phenology and ecology of color in bird-dispersed fruits, or why some fruits are red when they are" green". Can. J. Bot. 60: 701-713.

Summary in Japanese

タニウツギ属植物とフヨウ属植物における花色変化の生化学的機構と送粉昆虫に与える影響（英文）

多くの被子植物は開花した後に花色を変化させることはないが、開花後に経時的に花色を変化させる植物種も一部存在する。このような植物種では、どのようにして花色を変化させ、なぜこのような性質をもつように進化したのだろうか。本研究ではこれらの疑問を解決するために、花弁の色の変化の生化学的な機構を明らかにし、花色変化という性質が系統進化学的にどのように進化してきたのかを明らかにすることを目的として研究を進めた。それと同時に、花色変化という性質が送粉昆虫にどのような影響を及ぼすのか解明することも目的とした。

研究材料には、同属内で花色変化を起こす種が複数存在しているタニウツギ属 (*Weigela*) 植物とフヨウ属 (*Hibiscus*) 植物を用いた。タニウツギ属植物は東アジアに広く分布しており、世界におよそ 12 種が認められている (Hara 1983) 。その多くの種は赤色の花を付け、開花後に花色が変化することはないが、4 種では開花後に花色が白色から赤色へと変化することが知られている。フヨウ属は熱帯や亜熱帯を中心に 200 種以上が知られている。日本には花色が

開花後に黄色から赤色へと変化する種が 3 種知られている。

本論文の第一章では、まず、タニウツギ属植物の花色変化の生化学的機構を明らかにするため、植物色素の化学分析を行った。花弁に含まれる色素物質を同定するためにタニウツギ (124g) 、ハコネウツギ (107g) 、ニシキウツギ (86g) 、ウコンウツギ (156g)の花を用いて定性分析を行った。その結果、アントシアニンの 3 成分と、それ以外のフラボノイドの 5 成分が同定された。さらに、タニウツギ属植物 9 種 1 雑種の花弁に含まれる色素物質の定量分析を行った結果、他種とは異なり黄色の花をもつキバナウツギとウコンウツギを除き、色素組成にほとんど違いは見られなかった。また、花色変化する種としない種の間においても色素組成に違いは見られなかった。そこで、花色変化する種であるハコネウツギとニシキウツギ、および花色変化しない種であるタニウツギとヤブウツギの開花後の日数ごとに花弁に含まれる各色素の変化量を調べた。その結果、花色変化する種ではアントシアニン量のみが経時的に著しく増加し、花色変化しない種ではアントシアニンの量とその他のフラボノールの量はともに変化しなかった。したがって、この属の花色変化は、開花後の花弁に含まれるアントシアニンの増加によることが明らかになった。

次に第二章では、花色変化という性質がどのように進化したのか明らかにするために、タニウツギ属植物 12 種 1 雑種 2 品種について、核 rDNA ITS 領域の

塩基配列情報に基づく分子系統学的解析を行った。その結果、花色変化を起こす 4 つの種は 1 つのクレード（単系統群）にまとまらず、3 つの異なるクレードにおいて花色変化を起こさない種と混在した。このことから、花色を変化させる性質はタニウツギ属植物において複数の種群で平行的に進化したことが明らかになった。また、キバナウツギとウコンウツギは、その他のタニウツギ属の種と系統的に大きく離れていた。この 2 種は色素物質の組成も、その他の種とは大きく異なっていることから、タニウツギ属植物では花弁に含まれる色素物質の組成と分子系統樹による系統関係が対応していることが分かった。

さらに第三章では、花色変化するという性質が送粉者に及ぼす影響を明らかにするため、花色変化を起こす種であるハコネウツギ (*W. coraeensis*) 、およびその白花品種（白花のままで花色変化しない）であるシロバナハコネウツギ (*W. coraeensis f. alba*) が隣接して生育している自生地で訪花昆虫の調査を行った。調査は静岡県熱海市相の原町付近で行い、この場所では上記の 2 品種は 8m しか離れていない場所に自生していた。まず、送粉昆虫を決定するためにハコネウツギの訪花昆虫相、訪花頻度、一回訪花の結実率を調査したところ、ハコネウツギの送粉昆虫はコマルハナバチ (*Bombus ardens*) とジャコウアゲハ (*Byasa alcinous*) であることが分かった。そこで、ハコネウツギとシロバナハコネウツギの花に訪れるこれらの送粉昆虫の訪花個体数を比較・観察した。その結果、この植物種の

主要な送粉昆虫は花色変化するハコネウツギの花を有意に多く訪れていた。つまり、ハコネウツギは花色変化を起こすことで白花と赤花の 2 色の花色をもち、それによる色のコントラストが株をより目立たせ、遠距離からの訪花昆虫の誘因に役立たっていると考えられた。一方で、ハコネウツギの花色変化前の白花と花色変化後の赤花への送粉昆虫の訪花頻度を調査したところ、白花へ選択的に訪花していることが明らかになった。ハコネウツギの花色変化は、開花後に経時的に起こる。そのため、開花したばかりの白花は、開花後に数日経過した赤花に比べて未受粉である可能性が高いと考えられる。すなわち、ハコネウツギは主要な送粉昆虫に開花したばかりの白花に選択的に訪花してもらうことで、株全体の受粉効率を高めている可能性が示唆された。

最後に第四章ではフヨウ属植物の花色変化の生化学的機構を明らかにするため、植物色素の化学分析を行った。花弁に含まれる色素物質を同定するためにオオハマボウ (150g)の花を用いて定性分析を行った。その結果、アントシアニンの 1 成分と、それ以外のフラボノイドの 4 成分が同定された。さらに、花色変化する種であるハマボウ、オオハマボウ、テリハハマボウの花弁に含まれる色素物質の定量分析、ならびに開花後一定時間ごとに各色素の量を調べた。その結果、3 種において花弁に含まれる主要な色素物質の組成は全く同じであった。また、各色素の量を見てみると 3 種すべてでアントシアニンおよびその他

のフラボノイドの量がともに増加していた。特に、オオハマボウとテリハハマボウではアントシアニンの量の増加が著しいことが分かった。これらのことから、フヨウ属 3 種ではアントシアニンとその他のフラボノイドの量の増加によって花色変化が起きていることが明らかになった。

本研究によって、タニウツギ属植物における花色変化はアントシアニン量の増加によって起きていることが明らかになった。また、この属の花色変化という性質は複数の種群で平行的に進化したことが示された。そして、花色変化には色のコントラストによって遠くから送粉昆虫をより多く誘引する効果と、株内の若い花に送粉昆虫を誘導することで株の受粉効率を上昇する効果の 2 つがあることが分かった。一方、フヨウ属植物における花色変化はアントシアニン量とその他のフラボノイド量の両方の増加によって起きることが明らかになった。